

Growth of Lactic Acid Bacteria, with Special Reference to Meat and Meat Products

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LIST OF PAPERS

This thesis includes the following papers, (referred to in the text by their Roman numerals):

- I Blickstad, E., Enfors, S.-O., and Molin, G. 1981.
Effect of hyperbaric carbon dioxide pressure on the microbial flora of pork stored at 4 or 14°C.
Journal of Applied Bacteriology 50, 493-504.

- II Blickstad, E., and Molin G. 1983.
Carbon dioxide as a controller of the spoilage flora of pork, with special reference to temperature and sodium chloride.
Journal of Food Protection 46, 756-763, 766.

- III Blickstad, E., and Molin, G. 1983.
The microbial flora of smoked pork loin and frankfurter sausage stored in different gas atmospheres at 4°C.
Journal of Applied Bacteriology 54, 45-56.

- IV Blickstad, E., and Molin, G. 1981.
Growth and lactic acid production of Pediococcus pentosaceus at different gas environments, temperatures, pH values and nitrite concentrations.
European Journal of Applied Microbiology and Biotechnology 13, 170-174.

- V Blickstad, E.
Growth and end-product formation of two psychrotrophic Lactobacillus spp. and Brochothrix thermosphacta ATCC 11509^T at different pH values and temperatures.
Applied and Environmental Microbiology (in press).

- VI Blickstad, E., and Molin, G.
Growth and end-product formation of Brochothrix thermosphacta ATCC 11509^T and psychrotrophic Lactobacillus spp. in different gas atmospheres.

VII Blickstad E.

The effect of water activity on growth and end-product formation of two Lactobacillus spp. and Brochothrix thermosphacta ATCC 11509^T.

European Journal of Applied Microbiology and Biotechnology (in press).

SUMMARY OF PAPERS

The present thesis deals with the effect of environmental factors on the growth of meat associated lactic acid bacteria.

Studies on the natural flora

(papers I, II and III)

- High concentrations of carbon dioxide selected the microflora towards a domination of Lactobacillus spp. This was found for both lean and fat surfaces of pork, raw cured pork, smoked pork loin and sausage. Homofermentative Lactobacillus spp. dominated totally on the meat while on the heat treated meat products also a significant proportion of heterofermentative species were found in some cases.
- The effectiveness of the carbon dioxide in selecting the microflora towards Lactobacillus increased with decreasing temperature and increasing concentrations of CO₂. High concentrations of carbon dioxide and storage at low refrigeration temperature resulted in a very long shelf-life for the meat (about 80 days).
- The combination of curing and storage in a high CO₂ concentration at refrigeration temperature additionally reinforced the selection and furthermore prolonged the shelf-life.

- The shelf-life prolonging effect of high CO₂ concentrations on cured meat products varied with product type. A shelf-life of more than 7 months was obtained for frankfurter sausage (prinskorv), while no pronounced shelf-life prolonging effect was found for smoked pork loin.
- Many of the isolates of Lactobacillus spp. were unidentifiable with known species. Some isolates from the meat products which at first could not be identified at the genus level, could be identified as Lactobacillus after subsequent subculturing.

Studies on selected strains

(papers IV, V, VI and VII)

- Pediococcus pentosaceus was able to grow and produce lactic acid in N₂, CO₂ and air; at pH 15-45°C; at pH 5.3-7.3 and at nitrite concentrations ≤ 700 ppm.
- The lowest growth rates of the psychrotrophic Lactobacillus spp. and B. thermosphacta were found in pure carbon dioxide. The end-product formation was unaffected by the carbon dioxide.
- The growth rates of Lactobacillus viridescens and B. thermosphacta increased in the presence of oxygen. Oxygen induced the production of acetic acid, acetoin, i-valeric acid and hydrogen peroxide.
- The absence of oxygen drastically reduced the growth rate of B. thermosphacta.
- Only minor variations in the end-product formation of L. viridescens and the homofermentative Lactobacillus 173 were found due to the effect of pH, temperature or water activity.

- Reduced water activity or temperature affected the end-product formation of B. thermosphacta during aerobic growth.

INTRODUCTION

The natural habitats of lactic acid bacteria are plant material (grass, leaves, herbage), man and animals. In man and animals they are found for example in the intestinal tract and in the oral cavity (Sharpe 1981). Lactic acid bacteria have also found an ecological niche in foods. Their existence in connection with the activities of man may be beneficial or even necessary in one environment while detrimental in another.

Lactic acid bacteria are used by man in the production of many different types of fermented foods. Fermentation is, together with drying, the oldest method known for preserving foods. The characteristic aroma, flavour and texture of fermented foods are due to the growth of lactic acid bacteria. Furthermore, these bacteria exert a preservative effect through the production of for example acids, hydrogen peroxide or bacteriocins (Hurst 1973). Different genera are desirable in different types of foods.

In the dairy industry lactic acid bacteria are used in the manufacture of cheese, yogurt, kefir and cultured butter (Teuber and Geis 1981). Streptococcus (S. lactis, S. lactis ssp. cremoris, S. lactis ssp. diacetylactis, S. thermophilus), Lactobacillus (L. brevis, L. bulgaricus, L. helveticus) and Leuconostoc (Leuc. dextranicum, Leuc. cremoris) are used as starter cultures. The bacteria produce lactic acid from lactose whereby the pH is reduced down to a value of 4.0-5.6. Furthermore, aroma compounds such as diacetyl are formed and carbon dioxide may be produced. Finally the proteolytic enzymes produced contribute to the final texture of the food (Teuber and Geis 1981).

Lactobacillus and Leuconostoc develop spontaneously in wine which has a pH below 4.0. They convert malic acid to lactic acid and carbon dioxide which reduces the acidity of the wine, that is the pH increases (Sharpe 1981). This malo-lactic fermentation is both desirable and undesirable depending on the type of wine. Leuconostoc oenos is found in wine more frequently than Lactobacillus spp. (Sharpe 1981).

During the spontaneous fermentation of sauerkraut usually Lactobacillus and Leuconostoc develop (Stamer 1975). The same genera are also found in other fermented vegetables such as olives and cucumbers (Sharpe 1981). Factors that induce the development of lactic acid bacteria are tight packing of the vegetables and the addition of salt and sugar.

Silage based on grass, fish- or meat-wastes is preserved by lactic acid fermentation (Lindgren 1983). The growth of lactic acid bacteria is favoured by an anaerobic environment which may be obtained by tightly packing the raw material, and by the presence of fermentable carbohydrates. In a material with low carbohydrate concentration and a low initial concentration of lactic acid bacteria, carbohydrate and a starter culture are preferentially added. A starter culture based on Pediococcus acidilactici and Lactobacillus plantarum has been proven effective in the fermentation of silage (Lindgren 1983).

Lactic acid and dextran are two chemical products that are produced industrially by the use of lactic acid bacteria. Lactobacillus delbrueckii is used in the manufacturing of lactic acid (Lockwood 1979). Raw materials used are for example starch hydrolysate or dextrose syrups. The fermentation process takes place at 45-55°C for 5-10 days. Dextran is produced by Leuconostoc mesenteroides or Leuconostoc dextranicum from a medium containing sucrose (Kang and Cottrell 1979).

Lactic acid bacteria have been isolated from a variety of spoiled foods. In beer, heterofermentative Lactobacillus spp. and Pediococcus cause ropiness, off-flavours and acidity (Rainbow 1975). The storage life of vacuum-packed or gas-packed meat and meat products is usually limited by members of the genus Lactobacillus (Erichsen and Molin 1981; I; III). Typical defects are off-flavours, off-odours, discolourations or slime formation. Lactic acid bacteria may also cause spoilage in sugar refineries by the loss of sugar due to dextran formation. Lactobacillus and, especially Leuconostoc are associated with this type of spoilage (Tilbury 1975).

It can be concluded that lactic acid bacteria are found in many different habitats where they are of great financial significance.

The present thesis deals with the effect of environmental factors on the growth of meat associated lactic acid bacteria. Studies have been performed, both on the natural flora of lactic acid bacteria growing in competition with the total natural microflora (on meat or meat products; paper I, II and III) and on selected strains in pure cultures (in fermentor; paper IV, V, VI and VII).

CLASSIFICATION

Concept of lactic acid bacteria

In the classical monograph of Orla-Jensen (1919) the lactic acid bacteria are defined thus: "the true lactic acid bacteria form a great natural group of immotile, sporeless, Gram-positive cocci and rods, which in fermenting sugar chiefly form lactic acid". The five genera being included were Thermobacterium, Streptobacterium, Betabacterium, Streptococcus and Betacoccus. The first three genera now represent subgroups of Lactobacillus while

Betacoccus corresponds to Leuconostoc. Microbacterium and Tetracoccus (now Pediococcus) were not included in "the true lactic acid bacteria" by Orla-Jensen, since they were able to produce a catalase but were considered closely related.

Ingram (1975) characterized the lactic acid bacteria as being Gram-positive, catalase-negative, non-sporing, non-motile, acid-tolerant and fermentative lactic acid producers of non-aerobic environment and included Lactobacillus, Leuconostoc, Pediococcus and Streptococcus.

Recently, Kandler (1983) described the lactic acid bacteria as Gram-positive, non-sporing, microaerophilic bacteria that produce lactic acid as a major end-product from glucose. Kandler included Streptococcus, Pediococcus, Lactobacillus, Leuconostoc and Bifidobacterium. The inclusion of the Bifidobacterium can be questioned since this organism produces acetic acid and lactic acid in a molar ratio of 3:2.

However, as pointed out by Ingram (1975), the vague boundaries of the group result in that several other genera could be included in the lactic acid bacteria. Some examples of "presumptive" lactic acid bacteria are Brochothrix, Aerococcus, Listeria and Erysipelothrix. Brochothrix thermosphacta fulfil all given criteria for a lactic acid bacteria, except for having a variable catalase reaction (Davidson et al. 1968). However, also among the classical lactic acid bacteria an active catalase is found under certain conditions. Some strains of Pediococcus, Leuconostoc and Lactobacillus are able to split H_2O_2 when grown at low glucose concentration (0.05%) but not at high (1%; Whittenbury 1964). This type of catalase is called a pseudocatalase. Aerococcus share many characteristics with lactic acid bacteria, including the possession of pseudocatalase (Deibel and Seeley 1974). Another type of catalase, a heme-catalase, is produced by some Lactobacillus,

Leuconostoc, Pediococcus and Streptococcus if haematin is added to the growth medium (Whittenbury 1964). Erysipelothrix, another presumptive lactic acid bacteria was suggested as being included by Ingram (1975). However, Erysipelothrix is pathogenic and causes swine erysipelas (Ewald 1981). Apathogenicity was not mentioned as a characteristic of lactic acid bacteria by Orla-Jensen, Ingram or Kandler. This should, however, in my opinion, be an important criterion of a lactic acid bacteria since they are closely associated with use in foods. Listeria, finally, has a variable motility reaction in addition to being catalase variable. However, even among Lactobacillus motile strains may be found (Ingram 1975).

In a phenotypical numerical study which included several lactic acid bacteria, Microbacterium thermosphactum (now B. thermosphacta; Sneath and Jones 1976), Lactobacillus and Streptococcus were assembled into one of the main clusters (Wilkinson & Jones 1977). This cluster was closely associated with two other clusters including Listeria, Gemella and Erysipelothrix and it was suggested that these three genera together with B. thermosphacta, Streptococcus and Lactobacillus should all be included in Lactobacillaceae. Neither Pediococcus nor Leuconostoc were included in the study.

Studies of the immunological relationship between certain enzymes of lactic acid bacteria indicate that Lactobacillus is phylogenetically related to Leuconostoc, Pediococcus and Streptococcus, all genera sharing a common ancestor (London 1976). A high homology is also found between aldolase from B. thermosphacta and Streptococcus faecalis indicating relatedness. Furthermore a relationship, although more distant, seems to exist between Lactobacillus and anaerobes such as Eubacterium, Butyrivacterium and Propionibacterium.

In a recent study using oligonucleotide cataloguing of the 16S ribosomal RNAs of lactic acid bacteria, Pediococcus and Leuconostoc clustered together with the Lactobacillus in a phylogenetically coherent group, while the Streptococcus spp. did not (Stackebrandt et al. 1983). Streptococcus joined the lactobacilli cluster at the same level as Bacillus. According to Stackebrandt et al. (1983) "lactobacillus are an ancient grouping of organisms that together with bacilli and streptococci (and assorted others) arose from a common ancestor that was a clostridial type of organism".

Thus, the conception of Orla-Jensen of lactic acid bacteria as a group of closely related bacteria, is still valid in substance. The central genera are Lactobacillus, Leuconostoc, Pediococcus and Streptococcus. Several others may also be included since a strict definition of lactic acid bacteria cannot be made. For example B. thermosphacta is a presumptive lactic acid bacteria important in foods, especially meat and meat products. Considering how closely and often positively associated lactic acid bacteria are with foods, the apathogenicity should be an important criterion of these organisms. Additional criteria are the ability to produce major amounts of lactic acid and the Gram-positive reaction.

Identification

The genera Brochothrix, Lactobacillus, Leuconostoc, Pediococcus and Streptococcus can be separated by the characteristics given in Table 1.

Table 1. Characteristics of Brochothrix, Lactobacillus, Leuconostoc, Pediococcus and Streptococcus. All genera produce acid from glucose aerobically and anaerobically, are non-spore forming and non-motile. Tests are performed as described in paper I.

Genus	Morphology	Catalase reaction APT 20°C	Growth on STAA ¹⁾	Final pH, 2% glucose	Gas production from glucose
<u>Brochothrix</u>	rods	+	+	n.t. ²⁾	-
<u>Lactobacillus</u>	rods	-	-	n.t.	+/-
<u>Leuconostoc</u>	elongated cocci	-	-	n.t.	+
<u>Pediococcus</u>	cocci; pairs and tetraeds	-	-	<4.0	-
<u>Streptococcus</u>	cocci; pairs and chains	-	-	>4.0	-

1) Gardner 1966

2) n.t., not tested

Lactobacillus spp. are divided into homofermentative and heterofermentative species. The production of carbon dioxide from glucose is used as a differentiating test. Heterofermenters produce CO₂ while homofermenters do not (Rogosa 1974). A further subdivision of Lactobacillus into betabacteria, streptobacteria and thermobacteria (cf. Orla-Jensen 1919) may be done. Betabacteria is synonymous to heterofermentative species while strepto- and thermobacteria are homofermentative species. Streptobacteria may be separated from thermobacteria by the production of CO₂ from gluconate and the ability to grow at 15°C (Sharpe 1981). The ability to grow at 15°C is, however, not a particularly useful criterion since some thermobacteria are able to grow even at 5°C (Wilkinson and Jones 1977). It has been shown by Wilkinson and Jones (1977) that the subgroups streptobacteria, thermobacteria and betabacteria correspond to three different phenotypes of lactobacilli. Since this study many new Lactobacillus species have been de-

scribed and it could be called into question whether or not the separation between streptobacteria, thermobacteria and betabacteria is still useful.

The ability to produce acid from carbohydrates is used routinely to differentiate and identify species of Lactobacillus as well as of the other lactic acid bacteria (Sharpe 1979). The pattern of carbohydrate fermentations is often similar amongst different species with only a few tests differing. Practically, this means that a rather high number (about fifteen) of tests must be performed to increase the probability of including the separating test(s).

Fermentation patterns are not always correlated with relatedness between strains as shown by DNA - DNA homology studies, that is the correlation may be low between phenospecies and genospecies. Nakamura (1982) showed that four different strains of Lactobacillus amylophilus shared 90-98% DNA homology, even so the fermentation patterns were different. Lactobacillus lactis and Lactobacillus bulgaricus are separated by several fermentation reactions but the DNA homology is high (Dellaglio et al. 1973). Four different species of Lactobacillus (L. coryneformis ssp. torquens, L. delbrueckii, L. bulgaricus, L. helveticus) and L. amylophilus have very similar fermentation patterns, but in spite of this they are not related to each other, showing 6-15% DNA homology (Nakamura 1982). Thus, (1) genetically highly related strains may have different sugar fermentation patterns; (2) strains showing low relatedness may be difficult to separate with sugar fermentation patterns.

Recently, Leuconostoc mesenteroides, Leuconostoc dextranicum and Leuconostoc cremoris were brought together into one species, Leuc. mesenteroides, due to high DNA homology but despite having different phenotypic characteristics (Garvie 1983). The phenotypical differences are now only used for separation on the subspecies level.

Most heterofermentative Lactobacillus produce NH_3 from arginine and form DL-lactic acid from glucose which separates them from Leuconostoc (Sharpe 1979). However, some heterofermentative Lactobacillus are difficult to separate phenotypically from Leuconostoc. Lactobacillus viridescens and Lactobacillus confusus have similar sugar fermentation patterns to Leuconostoc and furthermore they are coccobacilli shaped just as Leuconostoc. L. viridescens resembles Leuconostoc further by not producing NH_3 from arginine and producing mainly D(-)lactic acid (Sharpe 1979). Electrophoretical patterns of glycolytic enzymes show great similarities between non-acidophilic Leuconostoc, L. viridescens and L. confusus (Garvie 1975). It has been speculated whether the heterofermentative Lactobacillus and Leuconostoc should be classified into one genera (Sharpe et al. 1972; Garvie 1975). However, RNA-DNA hybridization shows that L. confusus and L. viridescens are not closely related to Leuconostoc (Garvie 1981). Thus the problem with separating Leuconostoc and heterofermentative Lactobacillus by phenotypical characteristics still remains.

Many of the Lactobacillus spp. isolated from meat and meat products cannot be identified and grouped into the existing taxonomic system and terms such as "unclassified" or "atypical streptobacteria" are frequently used (Mol et al. 1971; Kitchell and Shaw 1975; Reuter 1975; Hitchener et al. 1982). In a study by Hitchener et al. (1982) none of 159 isolates of lactobacilli from vacuum packaged beef could be identified down to the species level. In a study on isolates from CO_2 -stored pork, on average 42% of the isolated Lactobacillus spp. were unidentifiable (Table 2; Blickstad, not previously published results). The unidentified strains constituted a heterogenous group regarding the phenotypical characters. Identified species included L. agilis, L. alimentarius, L. casei, L. mali, L. plantarum and L. "yamanashiensis" (Table 2).

Table 2.* Identification of Lactobacillus spp. isolated from lean or fat surfaces of native pork and from cured (3% NaCl) pork stored in pure CO₂ at 0°C and 4°C. The identification was based on sugar fermentation patterns in conventional test tubes and thirteen different carbohydrates were tested. MRS fermentation medium was used (Harrigan and McCance 1976).

Organism	Distribution (%)				
	Lean		Fat		
	0°C/79d	4°C/55d	0°C/79d	0°C/123d	4°C/67d
<u>Lactobacillus agilis</u>	15	0	0	0	0
<u>Lactobacillus alimentarius</u>	15	20	32	5	13
<u>Lactobacillus casei</u>	5	0	20	10	0
<u>Lactobacillus casei</u> ssp. <u>alactosus</u>	40	13	25	0	0
<u>Lactobacillus casei</u> ssp. <u>pseudoplantarum</u>	0	7	22	0	0
<u>Lactobacillus mali</u>	8	0	0	0	3
<u>Lactobacillus plantarum</u>	5	0	0	2	20
<u>Lactobacillus "yamanashiensis"</u>	8	0	0	0	0
<u>Lactobacillus</u> spp., unidentified	5	60	0	82	64
Number of isolates	40	15	40	40	30

* Blickstad, not previously published results.

MEAT AND MEAT PRODUCTS

Meat

The initial microflora of fresh meat has a heterogenous composition. Genera such as Acinetobacter, coryneform bacteria, Flavobacterium, Micrococcus, Moraxella and Pseudomonas are frequently found (Gardner et al. 1967; Enfors et al. 1979; I; II). The total aerobic count usually lies around 1000 organisms/cm² for fresh meat taken directly from the production line. The count of lactic acid bacteria is, however, generally below 10 organisms/cm² (Enfors et al. 1979; Christopher et al. 1980; I; II).

In spite of the great number of different genera found initially before storage, relatively few genera will become dominating and cause spoilage. Pseudomonas spp. will usually dominate on aerobically stored meat (Enfors et al. 1979; Erichsen and Molin 1981; I; II) while Lactobacillus spp. will dominate in vacuum packages (Enfors et al. 1979; Christopher et al. 1980; Erichsen and Molin 1981; I; II). High levels of B. thermosphacta have been found on products stored in vacuum packages and in air (Barlow and Kitchell 1966; Dainty et al. 1979; Erichsen and Molin 1981; II). A dominance of Leuconostoc spp. has seldom been reported, one occasion has been reported by Savell et al. (1981) for vacuum-packed beef. Thus, the storage life of vacuum-packed or gas-packed meat is usually limited by Lactobacillus, and occasionally by Brochothrix thermosphacta.

The presence of lactic acid bacteria may generally be regarded as positive. They are much less deleterious for the product quality than many other food spoilage bacteria such as Pseudomonas. Furthermore they grow relatively slowly. Together these two factors will lead to a longer shelf-life for the meat when the microflora is dominated by lactic acid bacteria. However, the lactic acid bacteria

will eventually spoil the meat. Lactic acid bacteria are able to produce, besides lactic acid, for example acetic acid, ethanol, acetoin, 2,3-butanediol, hydrogen peroxide, hydrogen sulphide or polysaccharides which may lead to spoilage (Sharpe 1962; Shay and Egan 1981; VI). Product deficiencies associated with lactic acid bacteria are off-odours, off-flavours, green discolourations and slime formation. The off-odours and off-flavours produced by lactic acid bacteria are described as "sour", "butter-milk", "acid", "liver-like" or "sulfur-like" (Egan and Shay 1982; Hanna et al. 1983). Some lactic acid bacteria have a pronounced spoilage ability. Among the lactic acid bacteria most commonly found on meat the spoilage ability decreases in the following order: Brochothrix thermosphacta > heterofermentative Lactobacillus spp. > homofermentative Lactobacillus spp. (Egan et al. 1980; Qvist and Mukherji 1981). Off-odour and off-flavour are noticed at an earlier stage for B. thermosphacta and heterofermentative spp. than for homofermentative spp. However, even among the relatively harmless homofermentative lactobacilli significant spoilers can be found, these for example being able to produce H_2S (Shay and Egan 1981) or H_2O_2 (VI).

Non-fermented cured meat products

The processing of meat products often includes curing, heat treatment, slicing and packaging. Before the heating process the number of bacteria may be high. During the heating the number is drastically reduced. In processed meats the total number of organisms per gram product is about 100 immediately after chilling (III). The initial microflora on fresh, heat-treated products is as heterogeneous as on meat and Arthrobacter, Bacillus, coryneform bacteria, Flavobacterium, Micrococcus and yeast have been found (Alm et al. 1961; Bell and Gill 1982; III). Immediately after the heat treatment, the number of lactic acid

bacteria and B. thermosphacta is low, often below 10 organisms/g product, but during the following slicing and packaging the product is recontaminated and their number increases. Luncheon meats were found to be recontaminated with up to 10^4 psychrotrophic organisms/g including about 30 lactic acid bacteria/g (Kempton and Bobier 1970).

Aerobically stored meat products may be spoiled by lactic acid bacteria, B. thermosphacta, Micrococcus, Vibrio, Acinetobacter and yeasts (Frazier 1967; Dowdell and Board 1968; Gardner 1971). Lactobacillus spp. often dominate the microflora of vacuum-packed meat products (Allen and Foster 1960; Alm et al. 1961; Mol et al. 1971; III). A dominance of Lactobacillus spp. has been reported for cured raw pork (II) as well as heat treated cured meat products (III) which have been stored in 100% CO₂. High levels of B. thermosphacta have been found on vacuum-packed cured meats (Shay et al. 1978; Qvist and Mukherji 1981; Steele and Stiles 1981) and cured raw pork stored in air (II). Leuconostoc have been found in high numbers on vacuum-packed meat products (Gardner and Patton 1971; Collins-Thompson and Rodriguez Lopez 1980), although this has not been frequently reported. Streptococcus have been found on vacuum-packed bologna but was probably due to temperature abuse during storage (Paradis and Stiles 1978). Investigations of vacuum-packed bacon stored at 20°C or 30°C revealed dominance of group D streptococci (Tonge et al. 1964). Furthermore, Streptococcus dominated on pasteurized luncheon meat stored at 25°C (Bell and Gill 1982). Pediococcus has not been reported as dominating on products stored at chill temperatures.

In conclusion, the microflora of vacuum-packed or gas-packed meat products is usually dominated by Lactobacillus spp. Occasionally B. thermosphacta or Leuconostoc spp. will dominate in vacuum-packages.

Fermented cured meat products

Fermented sausage is the most common type of fermented cured meat product. There is a great variety of types of fermented sausages and the processing procedures used. Three examples of typically Swedish fermented sausages are "Isterband", "medwurst" and "Sörgårdskorv". Isterband contains beef, pork (meat = about 37 weight %), fat, potato, barley-grain, curing salt, sugar, spices and ascorbic acid. After mincing and mixing the sausage mixture is stuffed into casings and smoked at 25-40°C for 15-20 hours. This type of sausage is eaten fried in a hot meal. Medwurst contains beef, pork (meat = about 75 weight %), fat, curing salt, sugar, salt and ascorbic acid. The smoking and fermentation process proceeds at 18-28°C for 3-8 days. Medwurst is eaten in thin slices on bread. Sörgårdskorv contains pork, beef (meat = about 52%), fat, potato flour, curing salt, sugar, blood protein, spices and ascorbic acid. The sausage is smoked and fermented at 30°C for 3 days. After the smoking process the sausage is heated to a centre temperature of 62°C. Sörgårdskorv is eaten sliced on bread. The temperature and time schedules for the smoking and fermentation processes are the ones recommended by the Swedish Farmers' Meat Marketing Organization but many local variations are to be found.

The fermentation process occurs simultaneously to the smoking. During the fermentation the growth of lactic acid bacteria is desirable. The lactic acid produced by the bacteria decreases the pH from the initial 5.8-6.4 down to 4.5-5.3 (Erichsen 1983). Furthermore a desirable texture and flavour develop. The growth of some types of lactic acid bacteria is unwanted since they produce carbon dioxide, acetic acid, slime or hydrogen peroxide, which lead to product defects. Desirable organisms are homofermentative Lactobacillus and Pediococcus.

The fermentation process is started spontaneously or by

the addition of a starter culture. The spontaneously started process involves chance inoculation, that is organisms present in the sausage mixture start the fermentation. This type of process may lead to product defects such as lack of flavour, soft-texture and rupturing of casings depending on that the wrong type of organisms have developed (Deibel et al. 1961). Lactobacillus and Pediococcus were found to dominate in spontaneously fermented sausages, but occasionally Bacillus or Streptococcus could dominate (Deibel et al. 1961).

In the starter culture process, a high concentration (about 10^6 organisms/g) of suitable organisms is added to the sausage mixture. After mixing, the sausage mixture is stuffed into casings and smoked. The use of a starter culture has several advantages over the spontaneous process such as reduced processing time and uniform colour, flavour, texture and pH (Everson et al. 1970). The method of using Lactobacillus as a starter culture in fermented sausage was patented by Jensen and Paddock in 1940. In 1959 the use of Pediococcus was patented (Niven et al. 1959). Since then many commercial starter cultures have been developed based on Lactobacillus and Pediococcus. Pediococcus pentosaceus is, for the time being, the most frequently used starter organism in Sweden.

ENVIRONMENTAL INFLUENCES ON GROWTH

Gas atmosphere

Packaging principles: The composition of the gas atmosphere surrounding a piece of meat or meat product is affected by the type of package used. The packaging principles used are aerobic storage, vacuum packaging and modified gas atmosphere. When storing aerobically a film with high permeability is wrapped around the product, thus maintaining an aerobic atmosphere. In a vacuum package the

gas volume is reduced by applying a vacuum before sealing the package. Both the meat and the bacteria present on the meat, consume the oxygen present and produce carbon dioxide (Ingram 1962; Gardner and Carson 1967). The small amount of oxygen in the vacuum package is thus reduced. The final concentration of oxygen attained depends on the film permeability. If the film is totally impermeable to air the oxygen level may be reduced to 0. In practice, small amounts of oxygen (<1%) may be present even if a film with a very low permeability ($10 \text{ ml O}_2/\text{m}^2/\text{day}$) is used (Erichsen and Molin 1981; III). The concentration of carbon dioxide in a vacuum package may increase up to 90% as reported for beef (Erichsen and Molin 1981). In the third type of package, the modified gas atmosphere, the air is replaced by a gas of known composition. The final gas composition may be somewhat different from the initial one due to air leakage through the package or to faulty welding seams (I; II; III). A wide variety of gases have been tested with variable O_2 -, CO_2 - and N_2 -concentrations (Clark and Lenz 1972; Huffman 1974; Christopher et al 1979; Enfors et al. 1979; Erichsen and Molin 1981).

CO_2 : Increased concentrations of CO_2 are known to inhibit many bacteria and the inhibitory effect increases with increasing CO_2 -concentrations. This has been demonstrated for the total spoilage flora on meat (Huffman 1974; Christopher et al. 1980; Erichsen and Molin 1981; I); and in pure culture studies of for example Bacillus cereus (Enfors and Molin 1980); B. thermosphacta (Gardner and Carson 1967; Roth and Clark 1975; Sutherland et al. 1977; VI); Escherichia coli (Molin 1983); Kurthia zopfii (Gardner and Carson 1967); Lactobacillus spp. (Sutherland et al. 1977; VI); Pseudomonas spp. (Sutherland et al. 1977; Enfors and Molin 1980); Yersinia frederiksenii (Molin 1983).

The inhibitory effect of CO_2 increases with decreasing temperature (Gill and Tan 1979; Enfors and Molin 1981).

Furthermore, the effect of CO_2 may be media-affected as reported for Pseudomonas fluorescens; the growth rate was reduced more by CO_2 in complex than in minimal medium (Gill and Tan 1979).

High levels of carbon dioxide have two major effects on the spoilage microflora of meat and meat products; (i) the flora is selected towards a total domination of Lactobacillus spp. and (ii) the growth rate of the Lactobacillus is reduced. As a consequence the shelf-life of products stored in pure CO_2 is prolonged as compared to storage in air or vacuum. This has been shown for beef (Erichsen and Molin 1981), pork (Enfors et al. 1979; I; II) and cured meat products (III).

In general lactic acid bacteria tolerate carbon dioxide well. Pediococcus pentosaceus and Streptococcus cremoris grow uninhibitedly in pure CO_2 (Enfors and Molin 1980; IV). Lactobacillus are tolerant to high CO_2 levels being able to out-compete other bacteria on meat even at 5 atm CO_2 (I). However, the growth rate of Lactobacillus spp. is reduced with increasing CO_2 ; raising the CO_2 level from 5% to 100% reduced the growth rate of a homofermentative Lactobacillus sp. by 22% and a heterofermentative by 10% at 25°C (VI).

The growth rate of B. thermosphacta decreases with increasing CO_2 -concentrations (Roth and Clark 1975; VI). On beef B. thermosphacta is totally inhibited by 100% CO_2 (Roth and Clark 1972; Sutherland et al. 1977). In culture media at pH 6.3 B. thermosphacta grows in the presence of 100% CO_2 although with a rate that is considerably lower than for Lactobacillus spp. (VI). This indicates that B. thermosphacta may be able to grow on CO_2 -stored meat provided that the pH is high enough, for example as for fat tissue, DFD and meat products. On high pH beef stored in 100% CO_2 the number of B. thermosphacta increased slightly from 2.2 to 2.5 log units/cm²,

while on normal pH beef the number decreased (Erichsen and Molin 1981). On fat tissue of pork stored in CO₂ the number of B. thermosphacta increased from < 1.0 to 3.2 log number/cm² (II).

A very low concentration of CO₂ is necessary for the growth of several lactic acid bacteria (Cogan et al. 1971; Repaske et al. 1974; VI). Furthermore, low concentrations of CO₂ are stimulative for several Lactobacillus spp. (5-10%; Rogosa 1974) and B. thermosphacta (3%, Gardner and Carson 1967).

O₂: Many lactic acid bacteria such as Lactobacillus casei (Erkama et al. 1968), Lactobacillus plantarum (VI), Lactobacillus viridescens (VI) and Pediococcus pentosaceus (Dobrogosz and Stone 1962; IV) grow well in air and the aerobic growth rate may even be higher than the anaerobic (VI). B. thermosphacta grows considerably faster aerobically than anaerobically (Brownlie 1966; Campbell et al. 1979; VI). In air the specific growth rate of B. thermosphacta at 25°C has been shown to be 0.47 h⁻¹, while it was 0.20 h⁻¹ in pure nitrogen (VI). In a batch culture, continuously sparged with air, the recorded O₂-tension was reduced to about 5% of saturation by B. thermosphacta. At this point the culture was in the logarithmic growth phase and the reduction in O₂ tension did not apparently reduce the growth rate. An oxygen concentration of 0.2% has been reported as sufficient for maintaining uninhibited growth rate of B. thermosphacta (Shaw and Nicol 1969). With increasing film permeability, that is with increasing availability of O₂, the growth of B. thermosphacta is enhanced (Newton and Rigg 1979).

pH

Red meat usually has a pH of between 5.4 and 5.8. DFD or high pH meat is in Sweden defined as a meat with a pH of

6.2 or higher. Fat tissue has a pH 6.8-7.2. The pH of cured meats varies but usually lies between 5.8 and 7.0.

Lactobacillus spp. have an optimum pH of 5.5-5.8 (Rogosa 1974). The growth of Lactobacillus spp. may be initiated at pH 3.8-7.2 (Corlett and Brown 1980). Leuconostoc spp. usually grow at pH 6.5 but not at pH 4.8 (Garvie 1974). Pediococcus pentosaceus grows at pH 4.3-7.3 (IV). B. thermosphacta grows aerobically at pH 5.0-9.0 with an optimum around pH 7.0 (Brownlie 1966). Anaerobically B. thermosphacta is unable to grow at pH 5.3 (VI). Furthermore the anaerobic growth on meat is totally repressed at pH <5.8 (Campbell et al. 1979). Undissociated L-lactic acid inhibits B. thermosphacta in anaerobic but not in aerobic environments (Grau 1980) and at pH 5.8 meat contains enough undissociated L-lactic acid to inhibit B. thermosphacta. At pH >6.0 B. thermosphacta grows anaerobically in culture medium (VI) as well as on meat (Campbell et al. 1979).

Water activity

The water activity ($a_w = \frac{p}{p_o}$; where p and p_o are the vapour pressures of solution and pure water, respectively) describes the amount of free water in a system. It may be lowered by drying, freezing or addition of solutes such as NaCl; methods which all are used in the preservation of foods. The water activity of fresh meat is about 0.99 and is reduced if the meat surface is dried. Cured meat products contain NaCl (1.5-3%) which gives an a_w of about 0.98. If the formula contains dry ingredients such as milk powder or soya protein the a_w may be reduced to about 0.96 (Leistner et al. 1980). Further reduction may be achieved by drying.

The minimum a_w for growth of bacteria is mostly above 0.90 a_w . Some minimum a_w values for bacteria are

reported in Table 3. The minimum a_w for lactic acid bacteria lies between 0.94 and 0.95. Thus, lactic acid bacteria may be considered as organisms being fairly tolerant to reduced a_w values as compared to other food-borne bacteria.

Table 3. Minimum a_w values permitting growth of some food-borne bacteria. The a_w was adjusted with NaCl.

Organism	a_w	Reference
<u>Pseudomonas fluorescens</u>	0.97	1
<u>Escherichia coli</u>	0.95	1
<u>Lactobacillus plantarum</u>	0.95	2
<u>Leuconostoc mesenteroides</u>	0.95	2
<u>Pediococcus pentosaceus</u>	0.95	2
<u>Salmonella newport</u>	0.95	1
<u>Brochothrix thermosphacta</u>	0.94	3
<u>Clostridium botulinum</u> , type A	0.94	1
<u>Staphylococcus aureus</u>	0.86	1
<u>Micrococcus</u> sp.	0.83	1

1 Marshall *et al.* (1971)

2 Troller and Stinson (1981)

3 Paper VII

The normal sodium chloride concentration used in cured meat does not repress the growth of B. thermosphacta and Lactobacillus spp. For example, on cured pork loin (3% NaCl) B. thermosphacta dominated the microflora in air while Lactobacillus dominated in CO₂ (II).

When other environmental factors such as pH, temperature and gas atmosphere are suboptimal the growth inhibitory effect of reduced a_w is enhanced (Scott 1957; Troller

and Christian 1978). The combination of curing and CO₂-storage gives meat an extended storage life compared to CO₂-storage alone (II).

Substrate

Low molecular weight substances that may be used by bacteria as a substrate for growth are abundant in meat. The concentrations of some such substances in meat are given in Table 4.

Table 4. Average concentrations of some compounds in meat that may be utilized as carbon and energy source.

Compound	Concentration (mg/g)	
	beef muscle tissue ¹⁾	lamb adipose tissue ²⁾
lactic acid	8.3	0.7
glycogen	3.2	
glucose-6-phosphate	2.7	
creatine	4.5	
carnosine	2.5	
inosine monophosphate	1.4	
glucose	1.1	0.04
taurine	0.6	
alanine	0.3	
anserine	0.3	
glutamate	0.3	
arginine	<0.05	
histidine	<0.05	
lysine	<0.05	
total amino acids	2.0	0.20
pH	5.5	7.2

1) 48 h post mortem; S. Fabiansson and A. Laser Reuterswärd, personal communication

2) Nottingham et al. (1981)

According to Gill and Newton (1978) glucose is probably preferentially used by bacteria as a substrate for growth

and when depleted the amino acids are attacked; the proteins are not attacked until a very late stage of spoilage. It has been suggested that the growth of the Gram-negative spoilage flora ceases before the amino acids are depleted, indicating O_2 limitation as a controlling factor (Gill and Newton 1977). While for the anaerobic Gram-positive spoilage flora the rate of diffusion of fermentable substrate to the meat surface determines the maximum cell density (Newton and Gill 1978).

Of the substrates available on meat for growth, B. thermosphacta utilize glucose and glutamate aerobically while only glucose anaerobically (Gill and Newton 1977; Newton and Gill 1978). Lactobacillus spp., isolated from meat products, may utilize arginine, glucose, glutamate, glycogen, histidine, inosine and α -ketoglutarate (Table 5).

Table 5.* Growth of Lactobacillus spp. on low molecular weight substances. In total 55 Lactobacillus isolated from meat or meat products were tested. Tests were performed anaerobically. Figures within parentheses give the number of positive strains.

Substance	Growth of <u>Lactobacillus</u>
L-alanine	-
L-arginine	+ (22)
L-carnosine	-
creatine	-
glucose	+ (55)
D-glutamic acid	-
L-glutamate	+ (1)
glycogen	+ (3)
L-histidine	+ (3)
inosine	+ (41)
α -ketoglutarate	+ (4)
L-lactic acid	-
L-lysine	-

* Blickstad, not previously published results

The concentrations of low molecular weight substances of adipose tissue is lower than that of meat (Table 4). Adipose tissue is spoiled in the same way as muscle tissue; amino acids are attacked after the glucose, and lipolytic activity is not necessary, according to Gill and Newton (1980). The low concentration of glucose indicates that fat tissue spoils more rapidly than muscle tissue (Nottingham et al. 1981). However, in the study by Blickstad and Molin (II) off-odours developed at the same time for both fat and for lean surfaces of pork. This was true both when stored in air and in pure CO₂.

Lactobacillus versus *B. thermosphacta*

The development of lactic acid bacteria and *B. thermosphacta* on vacuum-packed products often follows a similar pattern. Initially the count of lactic acid bacteria and *B. thermosphacta* increases. After one or two weeks of storage the growth of *B. thermosphacta* declines while the lactic acid bacteria continue to increase (Shay et al. 1978; Dainty et al. 1979; Collins-Thompson and Rodriguez Lopez 1980).

It is likely that the declination in *B. thermosphacta* occurs simultaneously with oxygen depletion as suggested by Gardner (1981) and the combined effect of anaerobic atmosphere and a pH ≤ 5.8 inhibits *B. thermosphacta*.

Some *Lactobacillus* such as *L. brevis* and *L. plantarum* are reported as inhibiting the growth of *B. thermosphacta* (Collins-Thompson and Rodriguez Lopez 1980; 1982). In these studies the pH was uncontrolled and the oxygen concentration was not measured. Thus, a combined effect of low pH and O₂ depletion cannot be excluded. In a recent study by Collins-Thompson et al. (1983) it was suggested that some inhibitory compound, so far unknown, was pro-

duced by L. brevis affecting the cell wall metabolism of B. thermosphacta and causing lysis. However, before any unambiguous conclusions can be drawn as to whether or not an inhibitory compound exists, it is essential when performing an experiment to have full control over environmental factors that are known to affect B. thermosphacta, for example pH, oxygen concentration and lactate concentration.

METABOLISM

Pathways of glucose fermentation

The energy requirement of lactic acid bacteria is met by the fermentation of organic substances, mainly carbohydrates. Lactic acid bacteria are divided into homofermentative or heterofermentative species depending on what type of end-products are formed from glucose: Streptococcus and Pediococcus are homofermentative while Leuconostoc is heterofermentative. Among the Lactobacillus both homofermentative and heterofermentative species are found.

The general pathway for the homofermentative species is the glycolytic pathway (Doelle, 1975; Figure 1). Lactic acid is the major end-product from glucose and about 1.6 moles lactate is formed per mol glucose consumed (VI). Small amounts of ethanol, acetic acid and formic acid may be produced additionally.

Heterofermentative species, such as Lactobacillus brevis and Leuconostoc spp., possess the phosphoketolase pathway (Doelle 1975; Mandelstam et al. 1982; Figure 2). About 1.0 mol lactic acid/mol glucose is formed (VI). In addition to lactate, carbon dioxide and ethanol or acetate are formed (Kandler 1983).

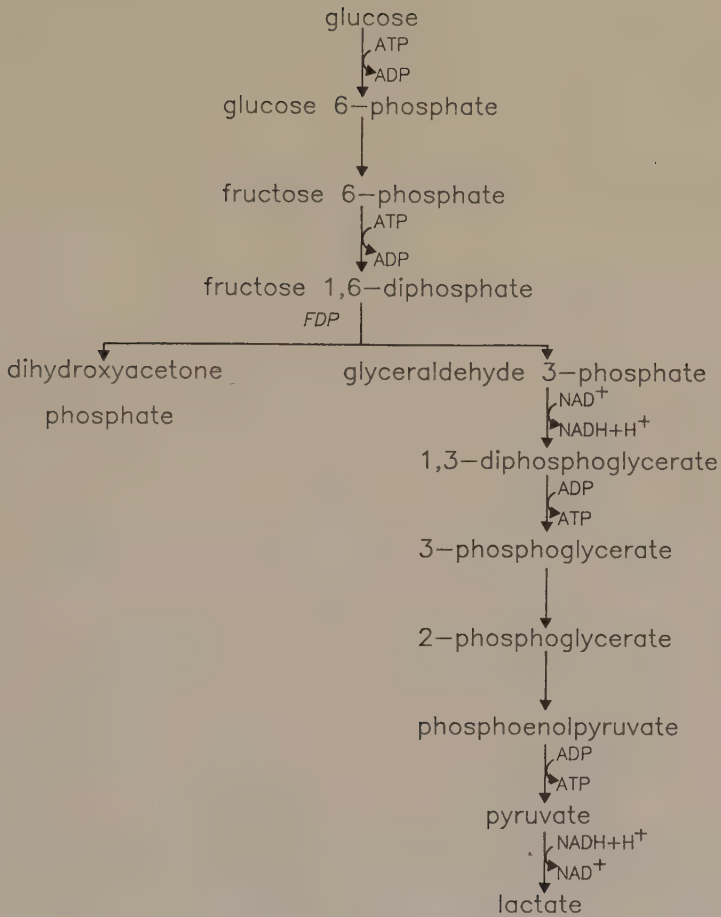


Figure 1

Homofermentative lactic acid bacteria metabolize glucose via the glycolytic pathway. (FDP = fructose 1,6-diphosphate aldolase).

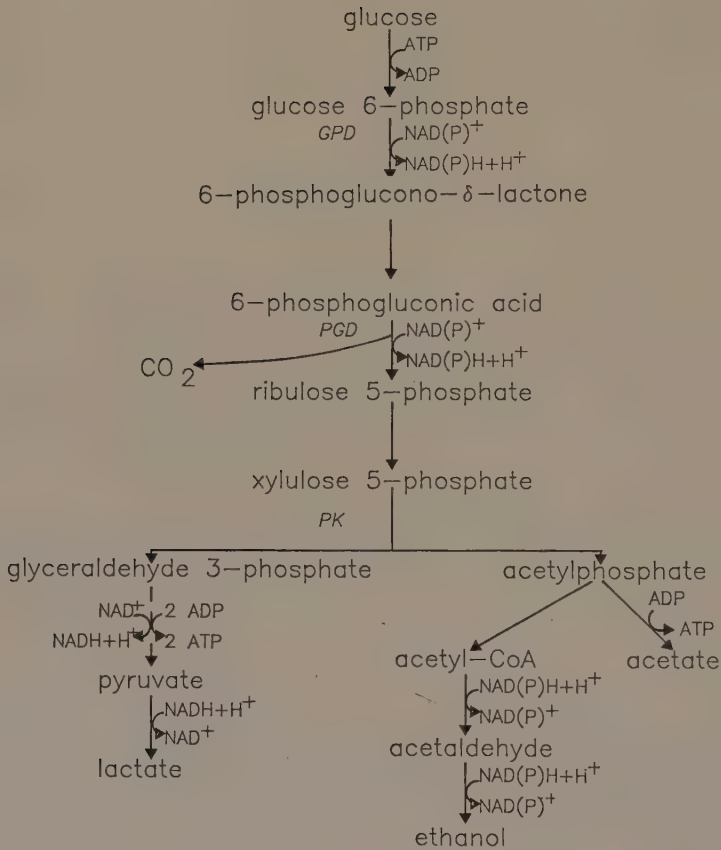


Figure 2

Heterofermentative lactic acid bacteria metabolize glucose via the phosphoketolase pathway. (GPD = glucose 6-phosphate dehydrogenase; PGD = 6-phosphogluconate dehydrogenase; PK = phosphoketolase).

The growth conditions may affect the amounts and types of end-products formed and end-products other than the above-mentioned may be formed. Some possible pathways for the dissimilation of pyruvate are outlined in Figure 3.

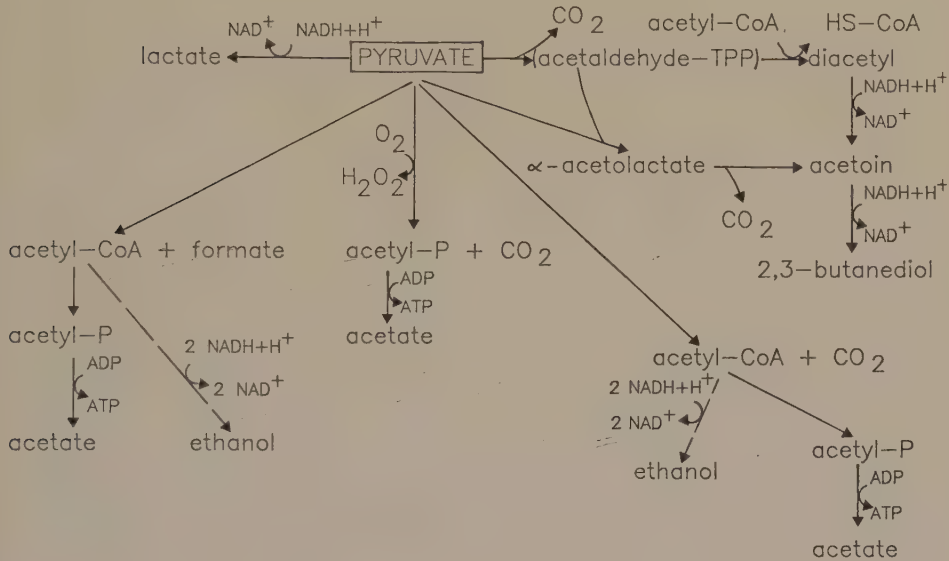


Figure 3

Dissimilation of pyruvate by lactic acid bacteria (Cogan *et al.* 1981; Mandelstam *et al.* 1982; Kandler 1983).

In the phosphoroclastic split a pyruvate lyase converts pyruvate into formate and acetyl-CoA and the latter is further metabolized into ethanol or acetate. By the action of a pyruvate dehydrogenase, pyruvate is split into acetyl-CoA and CO_2 . In the presence of O_2 , acetylphosphate and CO_2 are formed from pyruvate through the action of a pyruvate oxidase with the reduction of O_2 to H_2O_2 . Diacetyl, acetoin and 2,3-butanediol may be formed via pyruvate. Generally, much less diacetyl than acetoin is formed by the lactic acid bacteria (Collins 1972).

The metabolic pathway possessed by B. thermosphacta has not yet been fully investigated. B. thermosphacta possesses fructose 1,6-diphosphate aldolase (FDP), glucose 6-phosphate dehydrogenase (GPD) and 6-phosphoglucono dehydrogenase (PGD) as reported by Collins-Thompson *et al.* (1972). FDP is a key enzyme in the glycolytic pathway (Figure 1) while GPD and PGD are found in the phosphoketolase pathway (Figure 2). The presence of a phosphoketolase (PK, Figure 2), the key enzyme in the phosphoketolase pathway, has not been reported. In addition to L-lactic acid, B. thermosphacta may produce for example acetic acid, acetoin, *i*-butyric acid, ethanol, formic acid, 2-methylbutyric acid and *i*-valeric acid (Dainty and Hibbard 1983; VI). In a complex medium, acetoin and acetic acid are derived from glucose, while the precursors to *i*-valeric acid, 2-methylbutyric acid and *i*-butyric acid are leucine, isoleucine and valine, respectively (Dainty and Hibbard 1983). In a minimal medium *i*-valeric acid, 2-methylbutyric acid and *i*-butyric acid are formed from glucose by, so far, unknown mechanisms (Dainty and Hibbard 1983).

Alterations in metabolism

Gas phase: Under certain environmental conditions homofermentative lactic acid bacteria may become heterofermentative when growing on glucose. In air L. casei ssp. rhamnosus produces lactic acid and acetic acid while anaerobically no acetic acid is formed (Manderson and Doelle 1972). In the anaerobic environment the enzyme fructose 1,6-diphosphate aldolase (FDP) is active while aerobically the activity of FDP is reduced and in addition glucose 6-phosphate dehydrogenase (GPD) and 6-phosphoglucono dehydrogenase (PGD) are active. This means that in the presence of air "heterofermentative" enzymes may be induced in a homofermentative species.

Air (oxygen) also affects the metabolism of heterofermentative strains as reported for L. viridescens (VI) and Léuc. dextranicum (Keenan 1968). In air, the yield of acetic acid increases while the yield of ethanol and lactic acid decreases.

The metabolism of B. thermosphacta is highly affected by oxygen and the type of end-products formed aerobically are different from the ones formed anaerobically (Table 6). Aerobically B. thermosphacta produces acetic acid, acetoin, CO_2 , formic acid and i-valeric acid but no lactic acid. Anaerobically ethanol, formic acid and L-lactic acid are formed.

It is not unexpected that oxygen affects the metabolism since several lactic acid bacteria have respiratory activity. Oxygen is reduced by the action of a (NADH)oxidase (Anders et al. 1970). An active oxidase is reported for example for L. casei (Walker and Kilgour 1965; Brown and VanDemark 1968), B. thermosphacta (Collins-Thompson et al. 1972) and S. cremoris (Anders et al. 1970). The oxygen may be reduced to superoxide, hydrogen peroxide or water (Thomas and Pera 1983). Superoxide dismutase (SOD) and high internal levels of Mn^{2+} provide a defence against the toxic superoxide in several lactic acid bacteria and organisms devoid of SOD or Mn^{2+} are very sensitive to oxygen (Archibald and Fridovich 1981). Lactic acid bacteria decompose hydrogen peroxide through the action of peroxidase according to the reaction $\text{H}_2\text{A} + \text{H}_2\text{O}_2 \rightarrow \text{A} + 2\text{H}_2\text{O}$ where A may be e.g. NAD(P)^+ (Doelle 1975). The (NADH)peroxidase of L. casei is induced by oxygen and the enzyme activity is dependent on oxygen concentration (Erkama et al. 1968). The activity varies with the growth phase, and declines during the logarithmic phase (Erkama et al. 1968).

Thus, both the oxidase and peroxidase may be NADH-dependent. In the presence of oxygen less NADH is hence available in the catabolism than would be so in the absence of

Table 6. End-product formation of B. thermosphacta ATCC 11509T in aerobic and anaerobic culture.

Type of culture	mol%						CO ₂
	acetic acid	acetoin	ethanol	formic acid	L-lactic acid	i-valeric acid	
aerobic	34	56	0	3	0	7	+ ³⁾
anaerobic (N ₂)	0	0	9	4	86	0	- ²⁾

+, detected; - not detected

1) paper VI

2) Grau 1983

3) Blickstad, not previously published results

oxygen (Anders et al. 1970). The metabolic steps involving oxidation of NADH, that is the formation of lactate and ethanol, will be affected and the production of acetic acid and acetoin will instead be favoured.

pH: High pH may also change the metabolism. L. bulgaricus produces more formic acid, ethanol and acetic acid but less lactic acid at alkaline pH compared to acid pH (Rhee and Pack 1980). A corresponding trend is also reported for B. thermosphacta (Grau 1983).

Substrate: Several homofermentative Lactobacillus metabolize pentoses heterofermentatively to lactic acid and acetic acid (Williams 1975). A key enzyme in the heterofermentative pathway, pentose phosphoketolase, is induced in for example L. plantarum in the presence of pentoses (Williams 1975).

Low glucose concentration affects the metabolism of S. lactis, S. cremoris and L. casei (De Vries et al. 1970; Thomas 1979; Thomas and Turner 1981) diverting towards heterofermentative end-products.

Conditions such as low glucose concentration and low metabolic rate lower the fructose 1,6-diphosphate concentration. The type of lactate dehydrogenase, L(+)nLDH, that requires fructose 1,6-diphosphate in this case becomes inactivated (Garvie 1980). Most Streptococcus but only few Lactobacillus, (L. casei, L. curvatus) have fructose 1,6-diphosphate activated L(+)nLDH (Garvie 1980)

Water activity: Very little is known about whether or not or in what way the end-product formation is affected by reduced water activities. Troller and Stinson (1981) reported that lactic streptococci produced more diacetyl as the a_w was reduced. The end-product formation of L. viridescens was unaffected by the a_w , while the production of L-lactic acid by a homofermentative Lactobacillus was stimulated at low a_w values (VII).

CONCLUDING REMARKS

Many studies have been performed on lactic acid bacteria. The strains that have been given the greatest attention are the ones that are associated with dairy products, especially as starter cultures. However, the lactic acid bacteria are not only utilized in dairy products but also in a wide variety of other fermented food products. Furthermore, the lactic acid bacteria are important in the spoilage of many food products, for example prepacked meat and cured meat products. In spite of their great economic importance knowledge of the taxonomy, physiology and metabolism of the lactic acid bacteria is scanty and this is especially true for the non-dairy associated organisms.

There is a great number of different species of lactic acid bacteria. For example, 57 species of Lactobacillus have so far been validly published. The number, which has been more than doubled during the last ten years, is continuously increasing. In spite of the many described species it is frequently impossible to identify environmental strains of Lactobacillus. Furthermore, the G+C ratio varies from 34 to 53 mol% (Sharpe 1981), indicating that Lactobacillus today includes different genera.

It can be concluded that the present classification of lactic acid bacteria in general, and Lactobacillus in particular, needs revision. This is fundamental in order to be able to recognize and describe lactic acid bacteria found in different environments. Knowledge of the taxonomy is a reference point when performing ecological, physiological or metabolic studies. It is difficult to choose a relevant test strain if the taxonomic position is unknown. Furthermore, the knowledge of the taxonomy is essential in order to get a total survey of the biotechnical potential of a group of organisms.

At the establishment of refrigeration a couple of decades

ago, the shelf-life of perishable food products could be largely extended. Refrigeration storage can be made more efficient with a modified gas atmosphere around the product; that is with an increased carbon dioxide concentration. This is not a new idea (cf. Kolbe 1882) but it was not until the 1960's that the method was used on a wider, commercial basis with the vacuum packaging technique. Since then the use of a modified gas atmosphere is continuously increasing both as for wholesale and as for retail packages.

Smaller pork cuts are transported in 100% carbon dioxide from one factory to another within the Swedish Farmers' Meat Marketing Organization. The pork is transported in 500 l plastic containers at refrigeration temperature. Wholesale packages of 10 kg are also used for pork. Retail packages containing 20% CO₂ and 80% O₂ and different types of vacuum packages are being tested for beef and pork.

Some meat products, sausages and Swedish meat balls, are today distributed in retail packages with a controlled atmosphere containing a mixture of CO₂ and N₂. The purpose of this type of package is not to extend the shelf-life but to instead reduce dripping inside the package. In order to extend the shelf life of cured meat products a combination of reduced a_w values together with an atmosphere containing N₂ and CO₂ may be applicable in the future.

The advantage of storage in atmospheres having a high CO₂ concentration is not only the increased shelf-life. Additionally, a selection towards a Lactobacillus flora gives the product a "biological protection" against other spoilage organisms and pathogens. The protective effect of Lactobacillus is twofold; firstly by successful competition for growth space and secondly by a direct antagonistic effect. The best biological protection is obtained in

pure carbon dioxide at low refrigeration temperature applied when the product is packaged directly at the production line.

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PAPERS I-VII

Effect of Hyperbaric Carbon Dioxide Pressure on the Microbial Flora of Pork Stored at 4 or 14 °C

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Changes in the microbial flora of pork stored at 4 or 14°C were studied in 5 atm CO₂, 1 atm CO₂ or 1 atm air. The time needed for the total aerobic count at 4°C to reach 5×10^6 organisms/cm² was about three times longer in 5 atm CO₂ than in 1 atm CO₂, and about 15 times longer in 5 atm CO₂ than in air. At 14°C there was no difference in growth rate between 5 atm CO₂ and 1 atm CO₂. No off-odour was detected after storage in 5 atm CO₂ for 14 d, but the pork in 1 atm CO₂ (6 d) was organoleptically unacceptable.

The predominant organisms on the pork from the processing line were: *Flavobacterium* spp., *Acinetobacter calcoaceticus*, *Pseudomonas* spp., *Micrococcus* spp. and *Moraxella* spp. After aerobic storage at 4°C (8 d) or 14°C (3 d) more than 90% of the flora consisted of *Pseudomonas* spp. At 4°C all *Pseudomonas* spp. were of the non-fluorescent type, whilst at 14°C 32% were *Ps. putida* and *Ps. fluorescens*. After storage in 1 atm CO₂ *Lactobacillus* spp. represented 66% of the flora at 14°C (6 d) and 100% at 4°C (40 d), with *L. xylosus* dominating. After storage in 5 atm CO₂ *Lactobacillus* spp. constituted the total flora at both temperatures with *L. lactis* (14°C) and *L. xylosus* (4°C) dominating.

It was concluded that high partial pressures of CO₂ have a considerable shelf-life prolonging effect by (i) selecting the microflora towards *Lactobacillus* spp. and (ii) reducing the growth rate of these *Lactobacillus* spp. The controlling and growth inhibitory effect of CO₂ was promoted by reduced temperatures.

THE ABILITY of high concentrations of carbon dioxide (> 10%) to retard the growth of the Gram negative spoilage flora of meat, and in this way prolong the shelf-life, is well documented (Clark & Lentz 1972; Huffman 1974; Silliker *et al.* 1977; Sutherland *et al.* 1977; Christopher *et al.* 1979; Enfors *et al.* 1979). For example, Enfors *et al.* (1979) showed that the increase of the total count of bacteria on pork at 4°C was about seven times slower in 100% CO₂ than in air and that the microflora of the CO₂-stored pork was completely dominated by *Lactobacillus* spp., while on pork stored in air, *Pseudomonas* was the dominating genus. Thus, CO₂ provides an effective means of controlling the microbial flora of fresh, refrigerated meat.

Some investigators (Clark & Lentz 1969; Gill & Tan 1979), however, have reported a maximum partial pressure of CO₂ above which a further CO₂ pressure increase has but minor additional inhibitory effect. In contrast, other investigators (King & Nagel 1975; Enfors & Molin 1980) observed inhibitory effects which were approximately proportional to the partial pressure of CO₂ over the entire CO₂ pressure range.

These studies were all performed at CO₂ pressures in the range 0.1-1 atm (10.1-101 kPa) and no reports have been found dealing with the effects of hyperbaric CO₂ pressures on the microflora of meat. The intention of the present investigation was to

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compare the microbial development on pork stored in 5 atm CO₂ with that in 1 atm CO₂ or 1 atm air. The comparison has been made between 4 and 14°C.

Materials and Methods

Experimental design

Whole pork loins, cut in the conventional Swedish way (weight about 3.2 kg), were taken from the processing plant (Skanek, Kävlinge, Sweden) immediately after the butchery of the carcass. Before cutting, the carcasses had been refrigerated overnight (4–8°C) and the internal temperature of the meat had decreased to about 8–10°C. Selected loins had a normal visual appearance and the pH of the muscular tissue was 5.3 to 5.9. Each loin was packed in a plastic bag of Lamofol, *i.e.* laminated plastic of polyester (12 µm), aluminium (12 µm), polyamide (15 µm) and polythene (70 µm; Otto Nielsen Ltd., Denmark), or in high pressure vessels made of stainless steel (19.5 × 41 cm). The bags were filled with air (12 bags), or CO₂ (12 bags) and the pressure vessels with 5 atm of CO₂ (four vessels). The CO₂ (99.998%) was purchased from Alfax, Malmö, Sweden. The head-space of the plastic bags was 10 l and that of the pressure vessels about 8 l. The packed loins were stored at 4 or 14°C and subsequently withdrawn for microbiological sampling. Loins sampled once were not used again in the investigation.

Microbiological sampling

The method of Enfors *et al.* (1979) was used for the microbiological sampling of the loins. Conventional dilution procedures were used for the plate counts on Tryptone Glucose Extract Agar (TGE; Oxoid), acetate agar (AcA; Enfors *et al.* 1979), Violet Red Bile Dextrose Agar (VRBD; Difco) and Shahidi–Ferguson perfringens agar (SFP; Shahidi & Ferguson 1971). The TGE was incubated under aerobic and anaerobic conditions at 28°C for 3 d (total aerobic count and total anaerobic count) or at 5°C for 14 d (psychrotrophic count); AcA at 28°C for 5 d (lactic acid bacteria); VRBD at 37°C for 1 d (Enterobacteriaceae) and SFP incubated anaerobically (GasPak Anaerobic Systems; BBL) at 28°C for 7 d (sulphite-reducing clostridia).

Gas analyses

Before opening the packages for microbiological sampling, gas samples of 0.5 ml were taken with a gas-tight syringe and analysed for CO₂ and O₂ content. The analyses were performed with a gas chromatograph (Varian 3700) fitted with a thermal conductivity detector and a parallel system of 6 ft MS 5A (O₂) and 5 ft Porapak Q (CO₂) columns and with an empty column in series with the Porapak Q. Two samples were analysed from each package and the mean value of these analyses is presented.

Taxonomic classification

Taxonomic classification was performed on micro-organisms isolated from the differently stored loins. Isolates were picked from the countable TGE plate used for the

total aerobic count (28°C). From each plate (representing a single loin) about 20 colonies were picked using a random number generator and a matrix system applied to the plate (giving the destination of each colony).

A total of 374 isolates was classified. One hundred and twenty-two of these represented the initial flora of the pork (six loins) and the microbiological profile at the end of each storage situation was at each occasion represented by 38–45 isolates (two loins), except for the storage in 5 atm of CO₂ where each situation was represented by 42–45 isolates picked from a single loin.

The general key used for the taxonomic identification was *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons 1974) except for *Flavobacterium-Flexibacter* (Hayes 1977), *Brochothrix thermosphacta* (Sneath & Jones 1976), *Micrococcus* (Kloos *et al.* 1974) and *Staphylococcus* (Kloos & Schleifer 1975; Schleifer & Kloos 1975).

Tests used

All isolates were examined for Gram reaction; morphology and motility (phase-contrast microscope); pigmentation on TGE.

All Gram negative organisms were examined for aerobic and anaerobic breakdown of glucose (Hugh & Leifson 1953) and oxidase reaction (Kovacs 1956).

The Gram negative organisms were divided into *Pseudomonas* spp. (rods; motile; no anaerobic breakdown of glucose; oxidase positive), *Moraxella* spp. (rods; non-motile; no anaerobic breakdown of glucose; no pigmentation on TGE; oxidase positive), *Flavobacterium-Flexibacter* spp. (rods; non-motile; no anaerobic breakdown of glucose; pigmentation on TGE), Enterobacteriaceae (rods; anaerobic breakdown of glucose; oxidase negative) and *Acinetobacter* spp. (rods; non-motile; no anaerobic breakdown of glucose; no pigmentation on TGE; oxidase negative).

Pseudomonas spp. were examined for production of fluorescent pigment (King *et al.* 1954); DNase (Edwards & Ewing 1972); localization of flagella (Mayfield & Inniss 1977); hydrolysis of gelatin (Edwards & Ewing 1972).

Moraxella spp. were examined for deamination of phenylalanine (Edwards & Ewing 1972); haemolysis on blood agar [nutrient agar containing 7% (v/v) ox blood]; growth at 10°C on TGE; growth on surface of Hugh & Leifson medium; liquefaction of Loeffler's serum (Oxoid, PM200); production of urease (Edwards & Ewing 1972).

Flavobacterium-Flexibacter spp. were examined for spreading growth on cytophaga agar (Lewin & Lounsbury 1969); production of H₂S, indole and urease; growth on MacConkey Agar (Oxoid, CM115a); growth at 37°C in nutrient broth; acid formation from maltose, lactose in Hugh & Leifson medium; lipolysis of tributyrin (Hayes 1977).

Enterobacteriaceae were enumerated as described by Enfors *et al.* (1979).

Acinetobacter calcoaceticus was examined for production of catalase (Enfors *et al.* 1979), indole (Harrigan & McCance 1976), acetoin (Edwards & Ewing 1972).

All Gram positive organisms were examined for production of catalase in nutrient broth and on APT agar (Speck 1976) at 20°C; anaerobic growth in nutrient broth or MRS (deMan *et al.* 1960); acid production from glucose in MRS; growth on STAA (Gardner 1966).

The Gram positive organisms were divided into *Brochothrix thermosphacta* (rods; facultative anaerobes; catalase positive on APT agar at 20°C; growth on STAA; acid

production from glucose), *Listeria* spp. (coccoid rods; motile; facultative anaerobes; catalase positive), coryneform organisms (rods; facultative anaerobes or strict aerobes; non-motile; catalase positive; no growth on STAA), *Lactobacillus* spp. (rods; catalase negative; catalase negative on APT agar at 20°C; acid production from glucose in MRS), *Micrococcus* spp. (cocci; catalase positive; no production of acid from glycerol in the presence of erythromycin, P agar, Kloos *et al.* 1974), *Staphylococcus* spp. (cocci; catalase positive; production of acid from glycerol in the presence of erythromycin) and *Streptococcus* spp. (cocci; catalase negative).

Brochothrix thermosphacta was examined for production of acetoin, indole, oxidase; acid formation from sorbose, cellobiose, glucose anaerobically and aerobically in MRS.

Listeria spp. were examined for haemolysis on blood agar; aerobic and anaerobic breakdown of glucose in Hugh & Leifson medium; hydrolysis of Tween 80 (Sierra 1957); production of acid from mannitol, xylose, arabinose, sucrose in SBM medium (Wilkinson & Jones 1977).

Lactobacillus spp. were examined for gas production from glucose in Gibson medium (Gibson & Abd-el-Malek 1945) and growth in MRS at 15°C. Homofermentative organisms were examined for acid formation from amygdalin, cellobiose, galactose, lactose, mannose, raffinose, rhamnose, sorbitol, sucrose, trehalose. Heterofermentative organisms were examined for acid formation from lactose, mannitol, melezitose, melibiose, rhamnose, sorbitol, sucrose, trehalose and hydrolysis of arginine.

Micrococcus spp. were examined for growth in 7.5% NaCl on P agar; growth at 37°C on P agar; pigment production on P agar; hydrolysis of gelatin, Tween 80 and arginine (Edwards & Ewing 1972); production of acetoin (Edwards & Ewing 1972).

Staphylococcus spp. were examined for production of coagulase (Harrigan & McCance 1976); resistance to novobiocin (Kloos *et al.* 1974); acid formation from lactose, mannitol, mannose, trehalose (P agar); hydrolysis of arginine.

Streptococcus spp. were examined for production of gas from glucose in Gibson medium; terminal pH in nutrient broth with 2% glucose; growth at 10°C, 45°C, 50°C (TGE); growth in 6.5%, 8% NaCl (TGE); growth at pH 9.6 (TGE); acid production from glycerol, sorbitol, trehalose in Hugh & Leifson medium; haemolysis on blood agar.

Results

The bacterial growth on pork stored at 4 and 14°C in 5 atm CO₂, 1 atm CO₂ or 1 atm air, is summarized in Fig. 1*a* and *b*. The time needed for the total aerobic count to reach 5×10^6 organisms/cm² was at 4°C about three times longer in 5 atm CO₂ than in 1 atm CO₂ and about 15 times that in air. At 14°C the total aerobic count increased with almost the same rate in 5 atm CO₂ as in 1 atm CO₂. The growth rate was here one-third of that in air. It is also shown in the figure that the pork in 5 atm CO₂ at 14°C could tolerate a higher total aerobic count without the development of any off-odours.

It should be pointed out that the total aerobic count obtained on TGE at 28°C after 3 d was about the same as that at 5°C after 14 d. On the CO₂-stored loins (except 14°C for 1 d) the total anaerobic count was about the same as the total aerobic one. After storage in air the anaerobic count was generally lower than the aerobic count.

Table 1 shows the bacterial counts on different media. The growth of lactic acid

bacteria was stimulated by CO₂. The development of Enterobacteriaceae was retarded by 5 atm CO₂, especially at 4 °C, while 1 atm CO₂ had no profound effect as compared to air. There were no indications of growth of clostridia on meat stored in CO₂. However, several of the air-stored loins contained clostridia.

The amount of CO₂ and of oxygen in each bag and pressure vessel was analysed at the termination of the storage (Table 1). In four of the bags filled with pure CO₂ and in two of the pressure vessels small quantities of oxygen were found (0.1–1%). In the bags filled with air, CO₂ accumulated during the storage and after 8 d in 4 °C and 3 d in 14 °C the CO₂ concentration had reached 15%, i.e. about 0.05 l per kg pork and day was produced at 4 °C and about 0.15 l per kg and day at 14 °C.

The pH of the meat surface was not lowered by the CO₂ (Table 1).

The initial microflora of the pork had a highly variable composition (Table 2). The dominating organisms were, in order, *Flavobacterium* spp., *Acinetobacter calcoaceticus*, *Pseudomonas* spp., *Micrococcus* spp. and *Moraxella* spp. On average 14% of

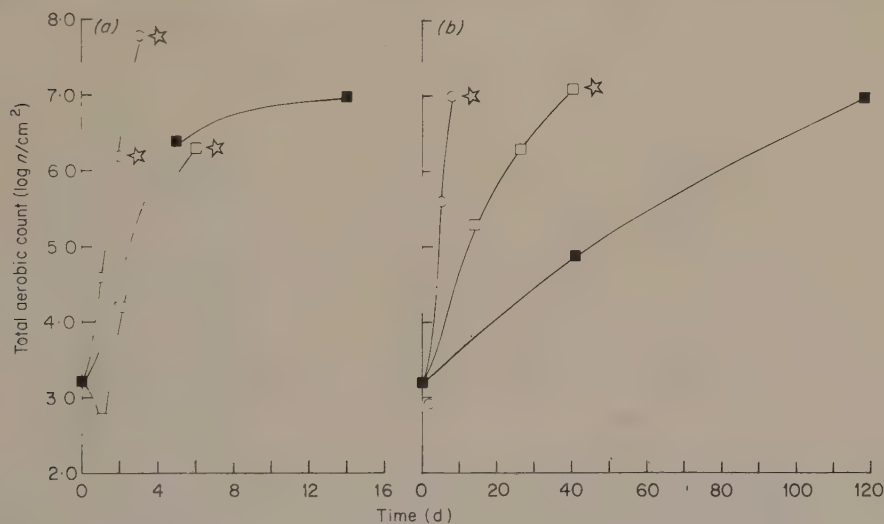


Fig. 1. Total aerobic count on pork stored in: ○, 1 atm air; □, 1 atm CO₂, and, ■, 5 atm CO₂ at (a) 14 °C and (b) 4 °C. The stars indicate loins which had developed off-odours.

the flora was *Pseudomonas* spp., but only 3% was of the fluorescent type. A very small number of *Brochothrix thermosphacta* was found.

The microflora of pork stored in air and at different CO₂ pressures at 4 and 14 °C is shown in Table 3. The flora of air-stored loins consisted at both temperatures of more than 90% *Pseudomonas* spp. The dominating part was non-fluorescent *Pseudomonas*. At 14 °C, however, there was also a considerable amount of fluorescent *Ps. fluorescens* (14%) and *Ps. putida* (18%).

On loins stored in CO₂ the microflora was dominated by *Lactobacillus* spp. (Table 3). *Lactobacillus* spp. dominated completely in all cases except one, i.e. after storage in 1 atm CO₂ for 6 d at 14 °C where 34% of the flora consisted of other genera. This loin also had a comparatively high percentage of heterofermentative *Lactobacillus* spp. (18%). The microbial profiles on the other CO₂-stored loins were relatively alike. On the loins stored at 4 °C (1 or 5 atm CO₂) *Lactobacillus xylosus* dominated (55–57%),

TABLE 1
Microbial counts on pork stored at different temperatures under different gaseous atmospheres

Initial gas atmospheres (atm)	Storage temperature (°C)	Storage time (d)	Analysed* CO ₂ (%)	Analysed* O ₂ (%)	pH	Total aerobic count TGE (log n/cm ²)	Lactic acid bacteria AcA (log n/cm ²)	Enterobacteriaceae VRBD (log n/cm ²)	Clostridia SFP (log n/cm ²)
—	—	0	—	—	5.6	3.2	0.2	0.5	0.0
1 air	4	2	1.9	21	5.6	2.9	0.9	0.2	0.0
1 air	4	5	5.3	17	5.6	5.6	1.4	2.5	0.0
1 air	4	8	15	5.1	5.6	7.0	2.7	4.4	2.4
1 air	14	1	4.8	20	5.8	4.6	0.8	2.8	0.4
1 air	14	2	6.6	19	5.7	6.2	2.4	5.3	1.5
1 air	14	3	15	7.0	5.5	7.8	4.1	5.7	0.7
1 CO ₂	4	14	100	0.0	5.7	5.3	5.4	2.4	0.0
1 CO ₂	4	26	98	0.1	5.5	6.3	6.0	3.3	0.0
1 CO ₂	4	40	96	0.7	6.0	7.1	6.5	5.5	0.0
1 CO ₂	14	1	95	1.0	5.6	2.7	0.3	0.2	0.0
1 CO ₂	14	2	100	0.0	5.6	4.2	1.9	3.7	0.5
1 CO ₂	14	6	99	0.1	5.5	6.3	6.1	5.4	0.0
5 CO ₂	4	41	98	0.6	5.9	4.9	4.8	0.0	0.0
5 CO ₂	4	118	100	0.0	6.6	7.0	6.9	1.1	0.0
5 CO ₂	14	5	99	0.2	5.7	6.4	6.2	5.3	0.6
5 CO ₂	14	14	100	0.0	5.7	7.0	7.0	<4.0	0.0

* At the time of opening.

Each figure represents the mean value from two different pork loins. n, Number of colonies. —, Not tested.

with unidentified homofermentative *Lactobacillus* spp. (24–26%). After storage at 14°C in 5 atm CO₂ the proportions of *L. xylosus* and of unidentified *Lactobacillus* spp. were small and instead the dominant group was *L. lactis*.

Discussion

In spite of the use of apparently gas-impermeable packaging materials and rigorous

TABLE 2

The microbial flora of pork taken directly from the processing plant

Organisms	% Organisms on different loins						Mean value
	1	2	3	4	5	6	
<i>Flavobacterium</i> phenon 3*	6	—	—	—	70	—	22
phenon 9*	10	—	5	5	—	21	
spp.†	—	9	—	—	5	—	
<i>Acinetobacter calcoaceticus</i>	—	30	33	25	—	26	19
<i>Pseudomonas</i> spp.							
fluorescent type	—	10	—	5	—	5	14
non-fluorescent type	6	14	—	27	5	11	
<i>Micrococcus kristinae</i>	10	14	18	9	—	5	13
varians	6	—	9	—	—	—	
luteus	—	—	—	5	—	—	
nishinomyaensis	—	—	5	—	—	—	
<i>Moraxella nonliquefaciens</i>	10	—	—	—	—	—	10
osloensis	17	—	—	—	—	—	
phenon 3†	10	—	—	—	—	—	
phenon 4†	6	—	5	—	5	—	
spp.‡	—	—	—	5	—	—	8
Coryneform group	6	10	15	5	5	5	
<i>Streptococcus faecalis</i>	—	—	5	5	5	—	6
spp.‡	6	—	5	—	—	11	
<i>Brochothrix thermosphacta</i>	—	—	—	5	—	—	1
<i>Listeria denitrificans</i>	—	5	—	—	—	—	1
<i>Providencia rettgeri</i>	—	—	—	5	—	—	1
<i>Staphylococcus capitis</i>	—	—	—	—	5	—	1
Yeast	6	9	—	—	—	16	5
Total aerobic count (log n/cm ²)	3.4	2.7	2.7	3.6	3.8	3.0	3.2

* Hayes (1977); † Thornley (1967); ‡ Unidentifiable.

precautions when packaging the pork loins, it was difficult to achieve a completely oxygen-free atmosphere in the sealed CO₂ packages. Only four packages out of 10 contained CO₂ without measurable amounts of oxygen (Table 1). Nevertheless, the oxygen concentration was low and never exceeded 1%.

In the pouches filled with air, there was a constant increase in the CO₂ concentration. At both storage temperatures the concentration reached 15%, which may have had a growth inhibitory effect on the total aerobic count. A level of 10% CO₂ is known to have a growth inhibitory influence on *Pseudomonas* spp. (Clark & Lentz 1969; King & Nagel 1975; Enfors & Molin 1980).

The shelf-life, expressed as the time needed for the total aerobic count to reach 5×10^6 organisms/cm², at 4°C, for example, was prolonged in 1 atm CO₂ by a factor of 5 and in 5 atm CO₂ by a factor of 15. If the shelf-life is measured by the time needed to develop off-odours, however, the prolonging effect of CO₂ is even higher which may be explained by the differences in microflora. Thus, high partial pressures of CO₂ have a considerable potential in preventing the spoilage of fresh meat. Furthermore, the growth inhibitory effect of CO₂ increased with decreasing storage temperature. Thus, the shelf-life of the air-stored pork was increased by a factor of three when the storage temperature was lowered from 14 to 4°C, while the shelf-life in 1 atm and 5 atm of CO₂

TABLE 3

The microbial flora of pork stored at different temperatures under different gaseous atmospheres

Organisms	% distribution of different organisms					
	Air		1 atm CO ₂		5 atm CO ₂	
	14°C/3 d	4°C/8 d	14°C/6 d	4°C/40 d	14°C/14 d	4°C/118 d
<i>Pseudomonas</i> spp.						
fluorescent type	32	—	—	—	—	—
non-fluorescent type	64	91	—	—	—	—
<i>Lactobacillus casei</i>	—	—	—	5	11	2
<i>curvatus</i>	—	—	—	3	—	5
<i>delbrueckii</i>	—	—	—	—	—	12
<i>lactis</i>	—	—	—	—	80	—
<i>plantarum</i>	—	—	3	3	—	—
<i>xylosus</i>	—	—	16	55	2	57
homoferment. spp.*	—	—	29	26	7	24
<i>cellobiosus</i>	—	—	3	—	—	—
<i>fermentum</i>	—	—	3	—	—	—
<i>viridescens</i>	—	—	12	8	—	—
<i>Brochothrix thermosphacta</i>	—	7	5	—	—	—
Coryneform group	—	—	10	—	—	—
<i>Micrococcus luteus</i>	—	—	3	—	—	—
<i>Enterobacter cloacae</i>	—	2	—	—	—	—
<i>Serratia liquefaciens</i>	4	—	16	—	—	—
Total aerobic count (log n/cm ²)	7.8	7.1	6.3	7.1	7.0	7.0

* Unidentifiable.

was increased by a factor of four and 15, respectively. The fact that low temperatures increase the growth inhibitory effect of CO₂ has been demonstrated before (Clark & Lentz 1969; Gill & Tan 1979; Enfors & Molin 1981). The present study demonstrates that this temperature effect is promoted by increasing CO₂ pressures.

High partial pressures of CO₂ may stimulate the germination of *Clostridium* spores (Enfors & Molin 1978). Nevertheless, there were no indications that the CO₂ atmospheres in the present study should support growth of *Clostridium* spp. on cold stored meat. Storage at high CO₂ partial pressures appeared to reduce the number of recoverable *Clostridium* present on the meat (Table 1).

The initial microflora of pork taken directly from the process line was dominated in

order by *Flavobacterium* spp., *Acinetobacter calcoaceticus*, *Pseudomonas* spp., *Micrococcus* spp. and *Moraxella* spp. Together these organisms constituted, on average, 80% of the flora. In a similar study on pork taken from the same slaughterhouse one year earlier, a similar pattern of the composition of the initial flora was noticed (Enfors *et al.* 1979).

The microflora on pork stored in air was, at both 4 and 14°C, dominated by non-fluorescent *Pseudomonas* (Table 3). After storage at 14°C, however, fluorescent *Pseudomonas* (32%) were also found. This supports earlier observations by Gill & Newton (1977), which showed that a non-fluorescent *Pseudomonas* sp. had a higher growth rate at temperatures below 15°C than a fluorescent one. In spite of the fast growth of non-fluorescent *Pseudomonas* spp. on the air-stored pork, there was a small but detectable number of Enterobacteriaceae present at both storage temperatures. This indicates that some members of Enterobacteriaceae have the ability to grow rapidly on cold-stored meat and if the growth conditions are altered in some way that is unfavourable to *Pseudomonas* spp. (e.g. vacuum packages), Enterobacteriaceae can assume a greater proportion of the microflora and may significantly contribute to the spoilage of the meat. One reason for the presence of *Enterobacter cloacae* and *Serratia liquefaciens* on the air-stored meat may be the gradual increase of CO₂ in the packages (Table 1).

The microflora of the CO₂-stored meat was in all cases dominated by *Lactobacillus* spp. (Table 3). Thus, high CO₂ concentrations were an effective means of directing the flora towards a domination of *Lactobacillus*. This has been demonstrated in earlier studies on pork stored in 1 atm CO₂ at 4°C (Enfors *et al.* 1979). The *Lactobacillus* flora in that study consisted after 35 d of mainly *L. plantarum* (45%) and *L. cellobiosus* (45%), while the flora in the present study, after similar storage conditions, was dominated by *L. xylosus* and a group of homofermentative but unidentified *Lactobacillus* spp.

A five-fold increase in the CO₂ pressure did not have any drastic effects on the composition of the microflora (Table 3), but the effect on the shelf-life was pronounced at 4°C (Fig. 1*b*). The growth rate of the *Lactobacillus* spp. was at 4°C strongly decreased, while the rate at 14°C was unaffected as compared to 1 atm CO₂. The low additional effect at 14°C may be due to the domination of *L. lactis* (only found on this loin), which perhaps may have a higher CO₂ resistance than the other *Lactobacillus* spp. In spite of this, it was clearly demonstrated that an increase in the CO₂ concentration generally decreased the growth rate of the *Lactobacillus* spp. on meat.

Another species of lactic acid bacteria, *Streptococcus cremoris*, has been tested for CO₂ resistance on a laboratory growth medium (Enfors & Molin 1980). This organism seemed to have a higher resistance to CO₂ than the majority of the *Lactobacillus* spp. found in the present study. The growth rate of *Strep. cremoris* was unaffected at 4.8 atm CO₂ and reduced to 50% at 8.6 atm CO₂. In the same report (Enfors & Molin 1980) the growth rate of *Ps. fragi* was reduced to 50% at 0.5 atm CO₂ and that of *B. cereus* at 1.3 atm CO₂.

Thus, all three of the organisms tested were inhibited by CO₂. The inhibitory effect started at different levels of CO₂ concentration and increased with increasing CO₂ concentration until the growth was stopped. These findings and the present investigation are not in accordance with statements such as 'concentrations above 20% appeared to be of little advantage' [referring to the effect of CO₂ on shelf-life of beef

(Clark & Lentz 1969)] and 'if other species respond similarly to CO₂ when growing on nutritionally complex substrates such as meat, this would explain why increasing the atmospheric content of CO₂ above 20% has little further inhibitory effect on spoilage flora growing at chill temperatures' (Gill & Tan 1979).

The present study together with other studies on the effect of CO₂ on the microflora of meat (Gardner *et al.* 1967; Beebe *et al.* 1976; Sutherland *et al.* 1977; Christopher *et al.* 1979; Enfors *et al.* 1979) indicate that the CO₂ concentration effects the microbial development on meat in two ways: (i) the CO₂ concentration applied selects the type of organisms that will dominate the flora and (ii) it affects the growth rate of these organisms. The tendency of the selection process on refrigerated meat at CO₂ concentrations around 0.2 atm seems to be that the inhibition of *Pseudomonas* spp. is relatively effective and that there is a shift in flora towards an increasing part of *Lactobacillus* spp. and also towards, for example, such organisms as *Brochothrix thermosphacta* and Enterobacteriaceae. At CO₂ pressures around 1 atm all organisms except *Lactobacillus* spp. are suppressed and if the CO₂ pressure is raised to a hyperbaric level, the growth rate of the *Lactobacillus* spp. is reduced and the shelf-life of refrigerated meat is further extended.

These results may aid the design of modern systems for the storage and transport of meat. Obviously, the higher the partial pressure of CO₂ the longer will be the shelf-life of the meat. A container system controlling the atmosphere with respect to CO₂ pressure, humidity and temperature, might permit the application of a hyperbaric pressure and thus enable the handling of fresh meat under conditions of minimum microbiological spoilage.

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Carbon Dioxide as a Controller of the Spoilage Flora of Pork, with Special Reference to Temperature and Sodium Chloride

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ABSTRACT

The microflora of native pork and cured pork (3% NaCl) stored in air or CO₂ (89-100%) at 0°C and 4°C were studied. The flora on native pork was followed both on fat and on lean surfaces. At 0°C the time taken for the lean pork to reach 10⁶ cfu/cm² was 6 times longer in CO₂ than in air (4 times at 4°C). The corresponding factor for cured pork was 10 at 0°C. The microflora was identified at the point of spoilage or just before. On lean and fat pork stored in air *Pseudomonas* spp. dominated. On fat significant amounts of *Alteromonas* spp. and *Brochothrix thermosphacta* were also found. The cured pork stored in air was dominated by *B. thermosphacta* while *Pseudomonas* constituted 20% of the flora. In CO₂ *Lactobacillus* spp. dominated to 100%, except on fat where *Alteromonas* spp. were also found. The combination of CO₂ and storage at 0°C suppressed growth of *B. thermosphacta*, *Enterobacteriaceae*, yeasts and molds. The study points to two practical implications: (a) storage in 100% CO₂ at 0°C gives the meat a shelf-life of about 3 months, (b) a combination of curing and storage in 100% CO₂ at 0°C gives the meat a shelf-life of more than 5 months.

The initial microflora of meat taken directly from the slaughtering line consists of a wide spectrum of different types of organisms in a nutritionally rich environment (1,7). However, that certain bacteria will grow and how fast they will grow, is dependant on the environment. The organisms best suited to the environment will be the most successful ones in the competition for living-space and will hence be the ones that will outgrow the others and dominate the spoilage flora.

High concentrations of CO₂ (20-100%) in the gas atmosphere around the meat have been proven to greatly influence development of the spoilage flora of refrigerated meat. Storage in CO₂ does not only prolong the shelf-life of meat by retarding microbial growth, it also selects for lactobacilli and inhibits pseudomonads (1,7,8).

However, several other environmental factors than the gas atmosphere will have an influence on the composition of the spoilage flora. These factors will be especially im-

portant for the capacity of the controlling effect of CO₂. The present study deals with the influence of temperature and NaCl on the microflora and shelf-life of refrigerated pork stored in 100% CO₂.

MATERIALS AND METHODS

Experimental design

Fresh pork loins with normal pH (5.4-5.8) were taken from the processing plant. Each pork loin was cut into two pieces (1.5 kg) and the loins were divided into three different groups: lean loins, all visible fat being removed; fat loins, a 1-2 cm fat layer covering the meat surface; cured loins, all visible fat being removed and the loins being stitch cured (Fomaco FGM 26, Garos AB, Västerås, Sweden) to a concentration of 3% sodium chloride and 180 ppm sodium nitrite. The lean and fat loins were packed immediately, while the cured loins were stored at 3°C overnight before they were packed. One piece of pork loin was put into each package. Loins stored in air were wrapped with a gas-permeable film (PVC film). The packaging material used for CO₂-storage of lean and fat loins was Mylothene/S (pvc lacquer/polyester/polyethylene film; permeability: 10 ml O₂/m²/day, 30 ml CO₂/m²/day; Otto Nielsen Ltd., Lyngby, Denmark) and for cured loins Lam-o-Foil 4 ply (polyamid/alufolie/polyamid/polyethylene film; permeability: extremely low; Otto Nielsen Ltd). The headspace in the CO₂ packages was about 3 L. The loins were stored at 0°C or 4°C and were subsequently withdrawn for analysis. Initially three pieces of lean, fat and cured loins were examined and during storage two pieces of lean, fat and cured loins were taken out on each occasion.

Microbiological sampling

Eight samples of meat were taken out with a cork borer (total sample area 25 cm²) from the surface of the boneless side of each piece of loin. Samples were pooled in 25 ml of physiological saline solution containing 0.1% (w/v) peptone and 0.1% (w/v) Tween 80 and then shaken for 30 min at 4°C.

Using conventional dilution procedures, viable counts were obtained on Tryptone Glucose Extract agar (TGE; Oxoid), Acetate agar (AcA; 7), Violet Red Bile Dextrose agar (VRBD; Oxoid), Potato dextrose agar (PD; Oxoid) and STAA (9). The TGE was incubated under aerobic and anaerobic conditions at 28°C for 3 d (total aerobic and total anaerobic count) or at 5°C for 14 d (psychrotrophic count); AcA at 28°C for 5 d (lactic acid bacteria); VRBD at 37°C for 1 d (*Enterobacteriaceae*); PD at 22°C for 5 d (yeasts and molds); STAA at 22°C for 2 d and then flooded with 1% tetramethyl-p-phenylenediaminedihydrochloride (*Brochothrix thermosphacta*; uncolored oxidase-negative colonies).

Gas analysis

The gas amount was examined as described by Blickstad et al. (1).

TABLE 1. Criteria used for the separation of gram negative rods into genera or family. Tests were performed as described by Blickstad et al. (1).

Genus, Family	Aerobic breakdown of glucose	Anaerobic breakdown of glucose	Motility	Oxidase	Pigmentation	Catalase	Resistant to pteridine 0/129 ^a
<i>Acinetobacter</i> spp.	v. ^b	—	—	—	—	+	nd
<i>Aeromonas</i> spp.	+ ^b	+	v.	+	—	nd	+
<i>Alteromonas</i> spp.	v.	—	+	+	v.	nd	nd
<i>Enterobacteriaceae</i>	+	+	v.	—	nd ^b	nd	nd
<i>Flavobacterium</i> spp.	v.	—	—	+	+	nd	nd
<i>Moraxella</i> spp.	— ^b	—	—	+	—	+	nd
<i>Moraxella</i> -like	+	—	—	+	—	+	nd
<i>Pseudomonas</i> spp.	v.	—	+	+	v.	nd	nd

^aCollins and Lyne, (4).^bnd, not determined; +, positive reaction; —, negative reaction; v, variable.

TABLE 2. Tests used for identification down to species level.

Organisms	Test	Reference
<i>Acinetobacter</i> spp.	See reference	1
<i>Aeromonas</i> spp.	Oxi/Ferm Tube (Roche), 2 d, 30°C	
<i>Alteromonas</i> spp.	See <i>Pseudomonas</i> spp.	
<i>Enterobacteriaceae</i>	Enterotube II (Roche) 1 d, 37°C + 2 d, 30°C	
<i>Moraxella</i> spp.	Growth TGE, 8°C	
<i>Moraxella</i> -like	Growth TGE, 8°C	
<i>Pseudomonas</i> spp.	Fluorescent pigment	13
	Hydrolysis of gelatin	5
	Acid from cellobiose, Hugh-Leifson medium	11
	Utilization of benzylamine, 2, 3-butanediol, mesoinositol, saccharate, trehalose	14
	Production of H ₂ S, TSI	5
<i>Yersinia frederiksenii</i>	Enterotube II; 1 d 37°C + 2 d, 30°C	
	Acid from melibiose, rhamnose, sucrose, D-xylose; Hugh-Leifson medium	
	Production of lecithinase	4
	Methyl red	5
<i>Brochothrix thermosphacta</i>	See reference	1
Coryneform bacteria	Hydrolysis of gelatin	
	Survival of heat treatment at 65°C for 10 min in nutrient broth	
	Acid from glucose, Hugh-Leifson medium	
<i>Lactobacillus</i> spp.	Gas from glucose; agar sealed MRS broth (Oxoid)	
<i>Micrococcus</i> spp.	See reference	1
<i>Staphylococcus</i> spp.	Production of coagulase	10
	Acid from glycerol in the presence of erythromycin, P agar	18

Identification

Isolates were picked from the countable TGE plates used for the total aerobic count. From each plate, representing one piece of pork loin, 10 colonies (initial microflora, 3 loins × 10 isolates) or 20 colonies (final flora, 2 loins × 20 isolates) were selected at random.

The general key used for the identification was *Bergey's Manual of Determinative Bacteriology* (3) except for *Alteromonas putrefaciens* (14), *B. thermosphacta* (21), *Pseudomonas* (14), *Staphylococcus* (18) and *Yersinia frederiksenii* (22).

All isolates were examined for gram reaction, morphology and motility (phase contrast microscope) and pigmentation on TGE.

The gram-negative isolates were divided into genus or family according to Table 1 and then examined further by using the tests listed in Table 2 or as described by Blickstad et al. (1).

The gram-positive isolates were divided into genera as described by

Blickstad and Molin (2). Gram-positive, catalase-negative cocci, not producing gas from glucose and with a terminal pH above 4.0 in nutrient broth with 2% glucose were considered to belong to *Streptococcus*. The isolates were examined further by using the tests listed in Table 2 or as described by Blickstad and Molin (2).

Off-odors

If any off-odors or discolorations were observed at the time of opening they were noted.

RESULTS

The microbiological development on pork loins stored at 0°C and 4°C in air or CO₂ is reflected by the bacterial

TABLE 3. Microbial counts, pH and gas concentrations of lean pork loins stored in air or CO₂ at 0°C or 4°C.

Gas atmosphere	Temperature (°C)	Storage time (days)	Total aerobic count (log n ^b /cm ²)	Total anaerobic count (log n/cm ²)	Lactic acid bacteria (log n/cm ²)	Brochothrix thermosphacta (log n/cm ²)	Enterobacteriaceae (log n/cm ²)	Yeast & mold (log n/cm ²)	pH	Analysed CO ₂ ^a (%)	Analysed O ₂ ^a (%)	Off odor
Air	0	0	2.2	1.4	<0	<1	<0.5	0.8	5.6	— ^c	—	no
		5	1.3	1.1	<0	<1.4	<0	0.5	5.4	—	—	no
		8	4.1	3.0	<0	<1.4	0.8	1.8	5.6	—	—	no
		15	6.0	4.8	<1.0	3.2	1.3	2.5	5.7	—	—	no
	4	20	7.7	6.5	<1.4	5.5	2.7	4.4	5.7	—	—	yes
		2	1.9	1.3	<0	<1	0	0.5	5.8	—	—	no
CO ₂	0	5	3.0	2.5	<0	1.5	<1.2	1.4	5.6	—	—	no
		8	6.2	4.6	<1.8	3.4	3.6	3.5	5.6	—	—	no
		12	8.2	6.8	2.4	5.7	5.4	4.4	5.8	—	—	yes
		13	1.8	1.3	<0	<1.0	<0	<0	5.6	98	0.0	no
		27	2.8	2.5	1.6	<1.0	<0	<0.4	6.0	98	0.0	no
		40	3.7	3.5	3.1	<1.0	<0.2	<0.4	6.1	100	0.0	no
	4	61	5.8	5.8	4.7	<1.0	<0	<0.2	6.3	97	0.1	no
		79	6.0	6.0	5.9	<1.0	<0.4	0.2	6.1	97	0.2	no
		119	6.9	7.0	7.0	<1.0	0.6	<1.4	5.8	97	0.0	yes
		13	2.2	2.1	1.2	<1.0	<0	<0	5.7	100	0.0	no
	27	5.8	5.9	5.2	<1.0	<0	0.2	6.0	100	0.0	no	
	40	6.3	6.4	6.0	<1.4	3.5	<1.0	6.0	99	0.0	yes	
	55	6.7	6.6	6.6	1.3	3.5	<1.0	5.9	99	0.0	yes	

^aAt the time of opening.
^bn, Number of colonies.
^c—, Not tested.

TABLE 4. Microbial counts, pH and gas concentrations of fat pork loins stored in air or CO₂ at 0°C or 4°C.

Gas atmosphere	Temperature (°C)	Storage time (days)	Total aerobic count (log n ^b /cm ²)	Total anaerobic count (log n ^b /cm ²)	Lactic acid bacteria (log n ^b /cm ²)	Brochothrix thermosphacta (log n ^b /cm ²)	Enterobacteriaceae (log n ^b /cm ²)	Yeast & mold (log n ^b /cm ²)	pH	Analysed CO ₂ ^a (%)	Analysed O ₂ ^a (%)	Off odors
Air	0	0	2.2	1.6	<0.3	<1.0	<0.4	0.7	6.8	— ^e	—	no
		5	3.4	3.0	<0.3	<1.1	<2.3	1.0	6.8	—	—	no
		8	4.2	3.0	<0.3	2.4	<0.2	1.4	6.4	—	—	no
		15	6.6	5.6	<1.0	4.2	1.0	2.7	6.1	—	—	no
		20	7.7	7.7	<0	5.7	3.2	4.0	6.6	—	—	yes
	4	2	2.3	2.0	<0.5	<1.0	0.4	0.7	6.5	—	—	no
		5	3.9	2.9	<0	1.9	1.8	0.8	6.3	—	—	no
		8	6.2	5.6	<1.4	3.0	3.5	3.7	6.5	—	—	no
		12	6.8	5.5	<0.5	4.4	4.1	3.8	6.6	—	—	yes
		13	1.6	1.6	<0.4	<1.0	<0.1	0.7	6.8	96	0.0	no
CO ₂	0	27	2.5	2.5	0.9	<1.0	<0	0.5	6.4	96	0.0	no
		40	2.7	2.7	2.1	<1.0	<0	<0	6.1	100	0.0	no
		61	4.4	4.3	1.7	<1.0	<0	<0	6.0	96	0.0	no
		79	5.0	5.1	4.2	<1.0	<0	<0	6.2	94	0.2	no
		119	5.4	4.9	4.7	<1.0	<0	<0	5.9	96	0.0	yes
	4	13	3.4	4.1	<0.9	<1.2	0.5	<1.0	6.6	94	0.1	no
		27	4.9	4.9	4.1	<1.5	1.8	0.8	6.2	93	0.1	no
		40	5.5	5.6	5.0	2.7	2.6	<1.0	6.1	93	0.0	yes
		55	6.4	6.6	6.7	3.2	3.7	0.8	6.5	89	0.0	yes

^aAt the time of opening.^bn, Number of colonies.^c—, Not tested.

TABLE 5. Microbial counts, pH and gas concentrations of cured pork loins stored in air or CO₂ at 0°C.

Gas atmosphere	Storage time (days)	Total aerobic count (log n ^b /cm ²)	Total anaerobic count (log n/cm ²)	Lactic acid bacteria (log n/cm ²)	Brochothrix thermophilacta (log n/cm ²)	Enterobacteriaceae (log n/cm ²)	Yeast & mold (log n/cm ²)	pH	Analysed CO ₂ ^a (%)	Analysed O ₂ ^a (%)	Off odors
Air	0	2.7	1.4	0.8	<1.0	0.5	1.4	5.7	— ^c	—	no
	4	2.8	1.6	0.3	<1.3	0.8	2.1	6.1	—	—	no
	7	2.5	2.4	<0	<1.3	<0	1.0	5.8	—	—	no
	14	2.6	0.8	<0	<1.0	<0	1.0	5.4	—	—	no
	32	5.8	4.8	3.2	3.8	<0	5.4	6.2	—	—	no
	39	6.7	6.4	2.2	5.4	<0	6.2	5.9	—	—	yes
CO ₂	19	2.2	2.3	<0	<1.0	<0	0.9	5.9	100	0.0	no
	39	2.6	2.3	2.2	<1.0	<0	0.5	5.8	100	0.0	no
	67	2.5	2.4	1.3	<1.0	0.6	0.2	6.2	100	0.0	no
	95	4.5	4.5	4.4	<1.0	<0	0.5	6.1	100	0.0	no
	109	5.5	5.5	5.5	<1.0	0.2	0.6	6.1	100	0.0	no
	123	5.1	5.0	4.4	<1.0	0.2	<0	6.1	99	0.1	no
	158	6.6	6.6	6.5	<1.0	<0	0.2	6.4	100	0.00	no

^aAt the time of opening.

Each figure represents the mean value from two different loins.

^bn, Number of colonies.^c—, Not tested.

count on different media as shown in Table 3 (lean surfaces), Table 4 (fat surfaces) and Table 5 (cured lean surfaces). *B. thermosphacta* was not detected on the loins stored in CO_2 at 0°C , while small numbers were found on uncured loins after storage in CO_2 at 4°C . In air, growth of *B. thermosphacta* occurred in fairly high numbers. The count of *Enterobacteriaceae* did not increase on the loins stored in CO_2 at 0°C nor on the cured loins stored in air. In all other instances an increase was found. Yeasts and molds were effectively suppressed by CO_2 .

The initial pH on the lean surfaces and on the cured lean surfaces was between 5.6 and 5.7. An increase in pH on these surfaces was found after storage in air and CO_2 . The initial pH on the fat surface was 6.8. The pH on the fat decreased in air and CO_2 and in the later gas atmosphere it decreased to 5.9 at 0°C .

The amount of CO_2 decreased in several packages with uncured loins and the lowest concentration found was 89% (Table 3,4). The packaging materials used for the cured loins were practically impermeable to air penetration and the concentration of CO_2 was 99-100% throughout the storage period (Table 5). Off-odors were detected in all packages at the end of storage except for cured loins stored in CO_2 at 0°C (Table 3,4,5).

The bacterial growth at 0°C on lean and fat surfaces and on cured lean pork stored in air or CO_2 is summarized in Fig. 1 a and 1 b. The time taken to reach 10^6 cfu/cm², for the lean loins, was about 6 times longer in CO_2 than in air. The corresponding factor was about 10 when comparing cured loins stored in CO_2 at 0°C with lean, uncured loins stored in air at 0°C , i.e. the curing reinforced the inhibitory effect of CO_2 . The total aerobic count of the fat loins, stored in CO_2 at 0°C , did not reach 10^6 cfu/cm².

The initial microflora of the pork had a highly variable composition (Table 6). The largest single group of organisms was *Micrococcus* spp. The cured loins also contained a high percentage of *Pseudomonas* spp.

The microbial flora after storage of fresh and cured pork in air and CO_2 is shown in Table 7. The flora was identified at a sampling occasion just before or at the point where the total aerobic count reached a level that gave rise to off-odors.

In air there was a general domination of *Pseudomonas* spp. on native pork. The proportion of *P. fragi* compared to *P. fluorescens* was slightly higher at 0°C than at 4°C . On fat surfaces the domination of *Pseudomonas* spp. was less pronounced and significant amounts of *Alteromonas* spp. (0°C) and *B. thermosphacta* (4°C) were found. The cured pork, after storage in air, was dominated by *B. thermosphacta* with *P. fragi* constituting only 20% of the flora (Table 7).

In CO_2 the microflora was dominated by homofermentative *Lactobacillus* spp. to 100% in nearly every instance (Table 7). The exception was on fat surfaces at 4°C , where *Lactobacillus* spp. constituted 83% of the flora while *Aeromonas* spp. constituted 15% of the flora.

In CO_2 the microflora was also identified at an earlier point when the total aerobic count was between 5×10^4

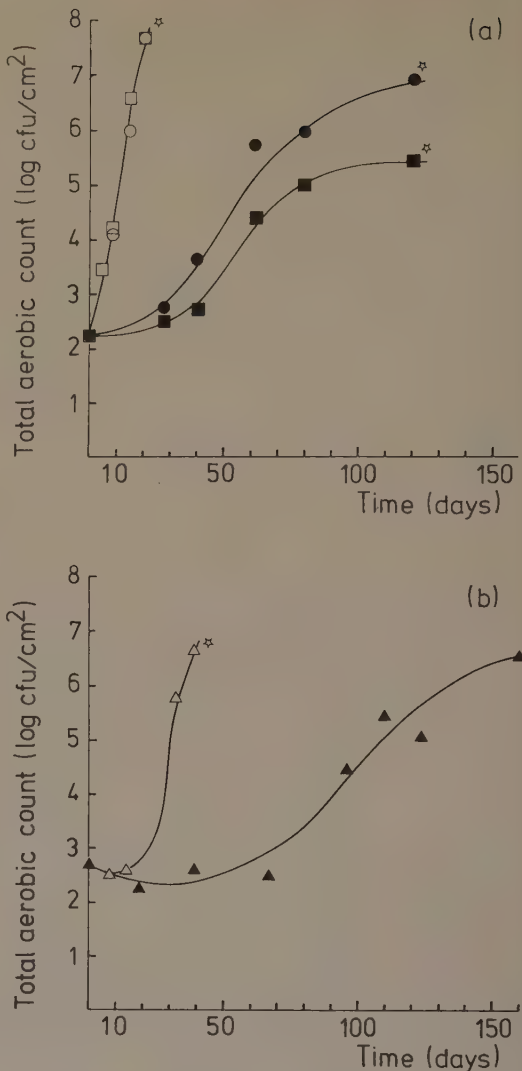


Figure 1. Total aerobic count on pork stored at 0°C in air or CO_2 ; (a) lean surfaces of fresh pork in (○) air or (●) CO_2 and fat surfaces in (□) air or (■) CO_2 ; (b) cured lean pork in (Δ) air or (▲) CO_2 . Stars indicate off-odor development.

and 5×10^5 cfu/cm². This corresponds to a storage time of 40 d (lean, fat, cured) at 4°C and 60 d (lean, fat), 95 d (cured) at 0°C . At this point the *Lactobacillus* spp. were already in an absolute majority on nearly all the tested pork loins. Some heterofermentative *Lactobacillus* spp. were found at this point representing 15% (lean, 0°C), 20% (fat, 0°C) and 25% (fat, 4°C) of the total flora. On one single loin (lean, 4°C) all of the 20 isolates were found to be *Y. frederiksenii* (total aerobic count: 10^5 cfu/cm²).

TABLE 6. The microbial flora on pork loins before storage.^a

Organism	Distribution (%)		
	Fresh		Cured
	Lean	Fat	
<i>Acinetobacter</i> spp.	0	7	3
<i>Aeromonas</i> spp.	0	0	7
<i>Brochothrix thermosphacta</i>	7	7	0
<i>Citrobacter freundii</i>	0	0	3
Coryneform bacteria	3	7	3
<i>Enterobacteriaceae</i>	3	0	3
<i>Escherichia coli</i>	0	3	0
<i>Flavobacterium</i> spp.	7	0	0
<i>Klebsiella</i> spp.	0	3	0
<i>Levinea amalonatica</i>	3	0	0
<i>Micrococcus</i> spp.	33	33	47
<i>Moraxella</i> spp.	10	7	0
<i>Proteus mirabilis</i>	3	3	0
<i>Pseudomonas</i> spp.	0	0	27
<i>Serratia liquefaciens</i>	3	0	0
<i>Staphylococcus</i> spp.	10	7	0
<i>Streptococcus</i> spp.	14	23	0
Yeast	3	0	7
Total aerobic count (log. n./cm ²)	2.2	2.4	2.7

^aEach figure represents a mean value from three different loins.

DISCUSSION

The CO₂-packed loins were meant to be stored under 100% CO₂. However, due to air penetration through the packaging (film material: pvdc lacquer/polyester/polyethylene), the CO₂ concentration in several packages decreased (down to 89%). According to earlier studies this may have created an environment more suitable for development of *Enterobacteriaceae*, *Aeromonas* and *B. thermosphacta* than would have been true in an absolutely pure CO₂ atmosphere (1,8,15).

The general trends of the present study are that CO₂ (a) strongly inhibits the increase in "total aerobic count" and (b) controls the microbial flora so *Lactobacillus* spp. become dominant. This is in full agreement with earlier studies on pork (1,7), beef (8) and fish (15).

The controlling effect of CO₂ was enhanced by a decrease in temperature from 4°C to 0°C. At 4°C the time taken to reach a total aerobic count of 10⁶ cfu/cm² was 4 times longer in CO₂ than in air while the corresponding factor at 0°C was 6, i.e. the relative shelf-life prolonging effect of CO₂ increased with the decreasing temperature. A similar effect was shown earlier in pure cultures of *Pseudomonas fragi* where the relative inhibitory effect of CO₂ on the maximum specific growth rate increased with decreasing temperatures (6). In the present study, the

TABLE 7. The microbial flora on pork loins after storage in air or CO₂ at 0°C or 4°C.

Organism	Lean				Fat				Cured	
	Air		CO ₂		Air		CO ₂		Air	CO ₂
	0°C	4°C	0°C	4°C	0°C	4°C	0°C	4°C	0°C	0°C
<i>Acinetobacter calcoaceticus</i>	—	—	—	—	—	2	—	—	—	—
<i>Aeromonas hydrophila</i>	—	2	—	—	—	—	—	—	—	—
<i>Aeromonas</i> spp.	—	2	—	—	—	—	—	15	—	—
<i>Alteromonas putrefaciens</i>	—	—	—	—	12	—	—	—	—	—
<i>Brochothrix thermosphacta</i>	—	—	—	—	—	18	—	—	60	—
Coryneform bacteria	—	5	—	—	—	—	—	—	—	—
<i>Enterobacteriaceae</i> , (unidentified)	5	—	—	—	5	—	—	—	—	—
<i>Lactobacillus</i> spp. (homofermentative)	—	—	100	100	—	—	100	83	—	100
<i>Moraxella</i> spp.	—	—	—	—	2	8	—	—	20	—
<i>Moraxella</i> -like spp.	2	—	—	—	—	2	—	—	—	—
<i>Pseudomonas</i> cluster 3 ^a	—	2	—	—	—	—	—	—	—	—
<i>Pseudomonas</i> cluster 4 ^a	—	2	—	—	—	2	—	—	—	—
<i>Pseudomonas fluorescens</i>	—	8	—	—	2	10	—	—	—	—
<i>Pseudomonas fluorescens</i> biotype 1 ^a	—	2	—	—	—	—	—	—	—	—
<i>Pseudomonas fragi</i> ^a	93	63	—	—	65	38	—	—	20	—
<i>Pseudomonas</i> spp.	—	8	—	—	12	20	—	—	—	—
<i>Serratia liquefaciens</i>	—	—	—	—	—	—	—	2	—	—
Unidentified	—	5	—	—	—	—	—	—	—	—
Number of isolates	40	40	40	20	40	40	40	20	40	40
Total aerobic count (log. no./cm ²)	6.0	6.2	6.0	6.7	6.6	6.2	5.0	6.4	6.7	5.1
Storage time (days)	15	8	79	55	15	8	79	55	39	123
CO ₂ (%)	nd ^b	nd	97	99	nd	nd	94	89	nd	99

^aMolin & Ternström (14).

^bnd, Not determined.

"total aerobic count" was represented by *Lactobacillus* spp.; hence, the impact of CO₂ in relation to temperature is valid also in respect to *Lactobacillus*. This temperature dependence is probably a general phenomenon and may be due to the increase in the solubility of CO₂ in water at decreasing temperatures. In fact, in the study by Enfors and Molin (6) this increase in water solubility accounted entirely for the temperature induced CO₂-effect in *P. fragi*.

Fat surfaces have been claimed to be more rapidly spoiled than lean ones (16). This is not in accordance with the results of the present study. On aerobically stored loins the increase in total aerobic count was the same for fat as for lean loins (Figure 1a). In CO₂ there was an even faster increase of the "total aerobic count" on the lean surfaces than on the fat surfaces. However, the time taken to develop off-odors was the same irrespective of the "total aerobic count."

In general terms, composition of the microbial flora was very similar, both on fat and lean surfaces (Table 7). This is consistent with results from lamb loins stored in air or in 20-40% CO₂ (20). However, in the present study there is a tendency towards increasing incidences of *Alteromonas putrefaciens* (air), *B. thermosphacta* (air) and *Aeromonas* (CO₂) on the fat surfaces. This result is probably due to the higher pH of the fat.

The growth of *Pseudomonas* is usually stopped at sodium chloride concentrations above 5% (12). The concentration applied in the present study was 3%. This decreased the number of *Pseudomonas* able to grow in air, but even so 20% of the flora was *P. fragi*. *Lactobacillus* spp. are less sensitive to NaCl than *Pseudomonas* and are usually able to grow in 6.5% NaCl (19). In the present study, the growth rate of the *Lactobacillus* spp. (representing the total aerobic count in CO₂) was strongly retarded on cured loins stored in CO₂. This retardation was probably not due to only the NaCl, since *Lactobacillus* is rather insensitive to the applied concentration. Instead it was probably due to the combined effect of NaCl and CO₂. This is in agreement with an early report by Rockwell and Ebertz (17), which indicated that salt "sensitizes" bacteria to CO₂. Thus curing enhances the shelf-life prolonging effect of CO₂.

The present study points to two major practical implications: (a) storage in 100% CO₂ at low refrigeration temperatures gives the meat a long shelf-life; about 3 months at 0°C, (b) a combination of curing and storage in 100% CO₂ at low refrigeration temperatures gives the meat an extremely long shelf-life; more than 5 months at 0°C. Furthermore, the study indicates that the more efficient the CO₂-storage is, the more efficient is the controlling effect of CO₂ on the composition of the spoilage flora, i.e. the ability to direct microbial load towards a pure *Lactobacillus* flora improves. During CO₂ storage at higher refrigeration temperatures there may be an increased risk of development of less desirable organisms, e.g. *Aeromonas* spp. and *Yersinia* spp. Apparently these organisms are almost as CO₂-resistant as *Lactobacillus* spp. and a small decrease in CO₂ concentration and/or an increase of some de-

grees in storage temperature (leads also to a lowering of the CO₂ concentration of the water phase) may facilitate a successful competition of these organisms towards *Lactobacillus* spp.

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The microbial flora of smoked pork loin and frankfurter sausage stored in different gas atmospheres at 4°C

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The development of the microflora of smoked pork loin and frankfurter sausage was followed during storage in vacuum, N₂ and CO₂ atmospheres at 4°C. The total aerobic count on the smoked pork loin reached 10⁷ organisms/g after 37 d in vacuum, 43 d in N₂ and 49 d in CO₂. The corresponding value for the sausage was 77 d in vacuum, while the growth stopped at 6 × 10⁴ organisms/g after 98 d in N₂, and at 4 × 10² organisms/g after 48 d in CO₂.

The predominant organisms on the fresh products were *Bacillus* spp., coryneform bacteria, *Flavobacterium* spp. and *Pseudomonas* spp.

At the end of the storage time the microflora on both products in the three gas atmospheres, consisted mainly of *Lactobacillus* spp. and two large groups of organisms that could not be identified as any described genus. Some of the unidentified strains could be classified as a *Lactobacillus* sp. after subsequent subculturing on laboratory media.

The numbers of *Lactobacillus* spp. at the end of storage decreased in the order, CO₂ > N₂ > vacuum. *Lactobacillus viridescens* generally constituted a substantial part of the *Lactobacillus* flora (5–72%). On the sausages two large uniform groups of unidentifiable homofermentative *Lactobacillus* spp. were also found.

The microbiology of raw meat stored in different gas atmospheres has been extensively studied. The shelf-life has been shown to decrease in the order, pure CO₂ > vacuum > N₂ > air (Enfors *et al.* 1979; Blickstad *et al.* 1981; Erichsen & Molin 1981). Carbon dioxide has a well-documented beneficial effect on the shelf-life of raw meat (Clark & Lenz 1972; Enfors *et al.* 1979; Blickstad *et al.* 1981). For example, Blickstad *et al.* (1981) demonstrated a five-fold increase in the shelf-life of pork loins when stored in pure CO₂, as compared with storage in air. This was explained by a shift from a *Pseudo-*

monas microflora to one dominated by *Lactobacillus* spp.

Studies on the microbiological development on processed meat have mainly been made on vacuum-packed products as vacuum packing is the usual way of prolonging the shelf-life of refrigerated meat products. A few studies have been made on meat products stored in N₂ or CO₂ (Callow 1932; Ogilvy & Ayres 1951; Ogilvy & Ayres 1953). The number of lactic acid bacteria on fresh processed meat is generally very low, and about 10 organisms per gram product have been found (Kempton & Bobier 1970). In spite of this the lactic acid bacteria dominate the flora in a vacuum pack (Allen & Foster 1960; Alm *et al.* 1961; Kempton & Bobier 1970; Mol *et al.* 1971), and are responsible for the spoilage of the product.

The intention of the present study was to determine how the microflora on two kinds of meat products were affected by vacuum, N₂ and CO₂ atmospheres.

Materials and Methods

EXPERIMENTAL DESIGN

The two products studied were smoked pork loin ('kassler') and a Swedish type of frankfurter sausage ('prinskorv'). During processing the pork loin was stitch-cured, smoked for 2 h and cooked for 1 h to an internal temperature of 65°C. The sausages were smoked for 40 min and cooked for 20 min to an internal temperature of 72°C. The sausages had the following formulation (kg): beef 24.0; pork 24.0; belly fat 20.0; potato flour 4.0; salt with nitrite 2.0; spices 0.5; ascorbic acid 0.02; water and ice 39.5. Both products were nitrite cured (smoked pork loin: 180 parts/10⁶ NaNO₂ added, final NaCl concentration about 2.5%; frankfurter: 120 parts/10⁶ NaNO₂ added, final NaCl concentration about 2.0%). The products were cooled overnight in a refrigerator at the processing plant and then packed in gas-impermeable film (smoked pork loin: PA80/PE100—PE100/PA80/PE100, Wipak, Nastola, Finland; sausage: Mylothene 12/80/S, Otto Nielsen, Lyngby, Denmark) with one piece of pork loin (450 g) or 12 sausages (280 g) in each pack. The pouches were evacuated (applied vacuum 600 Pa) and then either sealed directly (vacuum-packed) or filled with pure N₂ or CO₂. The headspace of the gas packs was about 0.7 l for the pork loin and about 1 l for the sausages. The products were stored at 4°C and were subsequently withdrawn for analysis. On each occasion two packs were examined, except during the initial sampling when four samples (4 × 450 g pork loin; 4 × 12 sausages) of each product were examined. In total, 28 samples of smoked pork loin and 36 samples of sausage were analysed.

MICROBIOLOGICAL SAMPLING

From the pork loin 5 wedges (2 cm deep, weight 30 g) were homogenized with 270 ml of physiological saline solution [plus 0.1% (w/v) peptone] in a Stomacher 400 (A.J. Seward & Co. Ltd., London) for 5 minutes. From the sausages ten slices (30 g) were cut and homogenized

with 270 ml of physiological saline solution for 2 min. The pH was measured in the homogenate.

Using conventional dilution procedures viable counts were obtained on Tryptone Glucose Extract agar (TGE; Oxoid), acetate agar (AcA; Enfors *et al.* 1979), Violet Bile Dextrose agar (VRBD; Oxoid), Shahidi-Ferguson Perfringens agar (SFP; Shahidi & Ferguson 1971), Potato Dextrose agar (PD; Oxoid), STAA (Gardner 1966) and *Yersinia* Selective agar (YS; Merck). The TGE was incubated under aerobic and anaerobic conditions at 28°C for 3 d (total aerobic and total anaerobic count) or at 5°C for 14 d (psychrotrophic count); AcA at 28°C for 5 d (lactic acid bacteria); VRBD at 37°C for 1 d (Enterobacteriaceae); SFP anaerobically (Gas Pak Anaerobic Systems; BBL) at 28°C for 7 d (sulphite reducing clostridia: black colonies); PD at 22°C for 5 d (yeast and moulds); STAA at 22°C for 2 d and then flooded with 1% tetramethyl-*p*-phenylenediaminedihydrochloride (*Brochothrix thermosphacta*: uncoloured, oxidase negative colonies) and YS at 25°C for 3 d (*Yersinia* spp.).

GAS ANALYSIS

The gas content in the gas-filled pouches was examined as described by Blickstad *et al.* (1981). The vacuum pouches were first filled with pure helium and then analysed in the same way as the gas-filled pouches.

DRIP

The drip, i.e. free meat juice in the pack, was weighed and calculated as a percentage of the sample weight.

IDENTIFICATION OF ISOLATES

Identification was performed on organisms isolated from the differently stored smoked pork loins and sausages. Isolates were picked from the countable TGE plates used for the total aerobic count (28°C). From each plate, representing the piece of smoked pork loin or the 12 sausages, 20 colonies were picked at random. A total of 200 isolates per type of product was classified; 80 of these represented the initial flora of the product. The microbiological profile at the termination of each storage period

(smoked pork loin: vacuum, CO₂ and N₂ after 48 d; sausage: vacuum 98 d, CO₂ 140 d, N₂ 140 d) was represented by 40 isolates (from two pouches).

The general key used for the identification was *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons 1974) except for *Arthrobacter*, *Kurthia*, *Microbacterium* (Jones 1975); *Brochothrix thermosphacta* (Sneath & Jones 1976); *Micrococcus* (Kloos *et al.* 1974); *Moraxella* (Thornley 1967); *Staphylococcus* (Kloos & Schleifer 1975; Schleifer & Kloos 1975).

All isolates were examined for Gram reaction, morphology and motility (phase contrast microscope) and pigmentation on TGE.

The Gram negative isolates were examined and divided into genera as described by Blickstad *et al.* (1981), except for *Pseudomonas*. *Pseudomonas* spp. were examined for growth at 4°C on nutrient agar (Oxoid).

The Gram positive isolates were divided into the different genera according to Table 1 and then examined further by using the tests listed in Table 2.

Unidentifiable *Lactobacillus* spp. were divided into two groups, group 1 (anaerobic growth; growth in MRS at 15°C but not at 45°C; no gas from glucose; acid from glucose; no acid from amygdalin, cellobiose, galactose, lactose, maltose, mannitol, mannose, raffinose, sucrose, trehalose or xylose; no hydrolysis of arginine) and group 2 [growth in MRS medium (Oxoid) at 15°C but not at 45°C; no gas from glucose; growth at pH 3.9; acid from amygdalin, cellobiose, galactose, maltose, mannitol, melibiose, ribose but not from gluconate, melezitose, raffinose, rhamnose, sucrose, trehalose or xylose; no hydrolysis of arginine].

Strains unidentifiable with any known genus, could be divided into two groups, group A (Gram positive rods; strict aerobes; catalase negative; not producing acid from glucose in MRS or Hugh & Leifson medium when incubated at 30°C for 7 d) and group B (Gram positive rods; strict aerobes; catalase negative; producing acid from glucose in MRS medium).

Results

The bacterial growth on smoked pork loin and on frankfurter sausage stored in different gas atmospheres is shown in Figs 1a and b. The

total aerobic count on the smoked pork loin (Fig. 1a), reached 10⁷ organisms/g after 37 d in vacuum, 43 d in N₂ and 49 d in CO₂. The corresponding value for the sausage (Fig. 1b) was 77 d in vacuum, while the growth stopped at 6 × 10⁴ organisms/g after 98 d in N₂ and at 4 × 10² organisms/g after 48 d in CO₂.

Table 3 shows the bacterial count of the smoked pork loins. The growth of lactic acid bacteria was stimulated in all gas atmospheres, but the number was less than the total aerobic count throughout the storage period. The highest count of yeast was found in vacuum at the end of storage. Small numbers of yeast were found in the N₂ flushed pack throughout the storage, but only in one pack containing CO₂. The largest number of *Brochothrix thermosphacta* was found in the presence of N₂, but the organism could be found in all three gas atmospheres. In N₂ a slight growth of *Broc. thermosphacta* was noted, but this growth declined during extended storage.

The psychrotrophic and the anaerobic counts were almost identical with the total aerobic count (Table 3). No Enterobacteriaceae, *Yersinia* spp. or clostridia were found (the count was below 10 organisms/g).

The amount of CO₂ increased to about 90% in vacuum packs and to 17% in N₂ packs (Table 3). Small quantities of O₂ (0.1–0.8%) were found in most of the pouches.

The pH of the smoked pork loin was not lowered in any of the gas atmospheres during storage (Table 3).

Drip was found in all vacuum packs and made up 5–6% of the sample weight (Table 3). Smaller amounts were found in the gas packages.

Table 4 shows the bacterial count in the sausages. The growth of lactic acid bacteria was stimulated in vacuum and N₂, while no increase in either lactic acid bacteria or the total aerobic count was found in CO₂. The number of lactic acid bacteria was considerably less than the total aerobic count in vacuum at the end of storage, and was equal to the anaerobic count. In N₂ and CO₂ no difference was found between the number of lactic acid bacteria, the total count and the anaerobic count. No difference was found between the psychrotrophic count and the total aerobic count in any of the gas environments. Yeast and mould were only found at the end of storage in vacuum (<2.3

Table 1. Criteria used for the separation of Gram positive isolates into different genera

	Rods	Cocci	Anaerobic growth	Catalase reaction, 30°C	Catalase reaction, APT 20°C	Growth on STAA	Acid from glucose, MRS	Endospores	Motile
<i>Arthrobacter</i> spp.	+		-	+	+	-	-	-	-
<i>Bacillus</i> spp.	+		-	nd	nd	-	-	+	nd
<i>Brochothrix (thermosphacta)</i>	+		+	-	+	+	+	-	-
<i>Corynebacterium</i>	+		-	+	nd	-	+	-	-
<i>Erysipelothrix</i> spp.	+		+	-	-	-	-	-	-
<i>Kurtzia (zopfii)</i>	+		+	+	+	-	-	-	-
<i>Lactobacillus</i> spp.	+		-	+	-	-	+	-	-
<i>Leuconostoc</i> spp.	elongated	cocci	+	-	-	-	+	-	-
<i>Listeria</i> spp.	+		+	+	+	-	+	-	+
<i>Microbacterium</i> spp.	+		+	+	+	-	+	-	-
<i>Micrococcus</i> spp.		+	nd	+	nd	nd	nd	-	-
<i>Staphylococcus</i> spp.		+	nd	+	nd	nd	nd	-	-

Tests were performed as described by Blickstad *et al.* 1981.

+, Positive reaction.

-, Negative reaction.

nd, Not determined.

Table 2. Tests used for identification of Gram positive strains down to species level

Organism	Test	Reference
<i>Arthrobacter</i> spp.	Acid from glucose; Hugh-Leifson medium Hydrolysis of gelatin	Hugh & Leifson 1953 Edwards & Ewing 1972
<i>Bacillus</i> spp.	Growth at 3°C, 55°C; nutrient broth Swelling and position of spores Acid and gas from glucose; Medium 1 Acid from arabinose, xylose; Hugh-Leifson medium Deamination of phenylalanine, urea Hydrolysis of gelatine, starch Lecithinase activity Haemolysis on blood agar; nutrient agar with 7% oxblood Proteolysis of casein Production of acetoin	Gibson & Gordon 1974 Edwards & Ewing 1972 Edwards & Ewing 1972, Collins & Lyne 1970 Collins & Lyne 1970 Harrigan & McCance 1976 Edwards & Ewing 1972
<i>Brochothrix thermosphacta</i>	As described by Blickstad <i>et al.</i> 1981	
<i>Erysipelothrix</i> spp.	Acid from arabinose, cellobiose, fructose, mannitol, trehalose, xylose; SBM-medium	Wilkinson & Jones 1977
<i>Kurthia zopfii</i>	see <i>Arthrobacter</i> spp.	
<i>Lactobacillus</i> spp.	Acid from amygdalin, cellobiose, galactose, lactose, maltose, mannose, melezitose, raffinose, sucrose, trehalose, xylose; MRS fermentation medium Hydrolysis of arginine Production of slime from sucrose Growth at 5°C, 15°C, 45°C; MRS Gas from glucose; Gibson medium	Harrigan & McCane 1976 Collins & Lyne 1970 Harrigan & McCance 1976 Oxoid Gibson & Abdel-Malek 1945
<i>Leuconostoc</i> spp.	see <i>Lactobacillus</i>	
<i>Listeria</i> spp.	As described by Blickstad <i>et al.</i> 1981 Reduction of nitrate; medium modified by omitting the agar and the presence of nitrite was tested with Merchoquant test strips, Merck	Edwards & Ewing 1972
<i>Microbacterium</i> spp.	Acid from fructose, glucose, maltose; Hugh-Leifson medium Survival of heat treatment at 65°C for 10 minutes; nutrient broth	
<i>Micrococcus</i> spp.	As described by Blickstad <i>et al.</i> 1981	
<i>Staphylococcus</i> spp.	Production of coagulase Resistance to novobiocin Acid from cellobiose, maltose, mannitol, raffinose, sucrose; P agar	Harrigan & McCance 1976 Kloos <i>et al.</i> 1974 Kloos <i>et al.</i> 1974

log n/g). No *Broc. thermosphacta*, Enterobacteriaceae, *Yersinia* spp. or clostridia were found (count below 10 organisms/g).

The amount of CO₂ increased in vacuum to 21%, while no increase was found in N₂ (Table 4). Small amounts of O₂ were detected in some of the pouches.

The pH was initially 5.9 (Table 4). A slight decrease was found after storage in vacuum and CO₂.

Drip was found in the vacuum and CO₂ pouches, but seemed to decrease at the end of storage. No drip was found in pouches containing N₂.

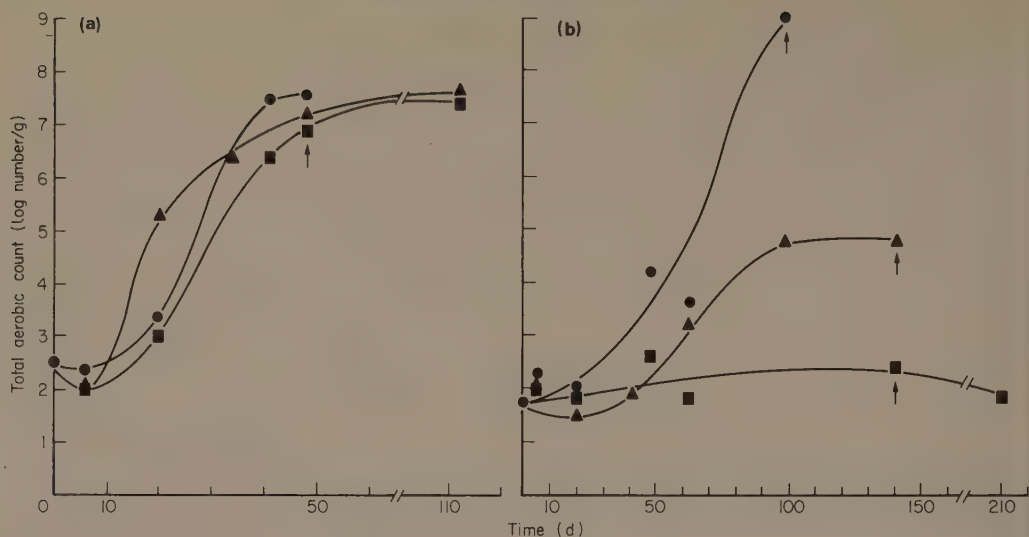


Fig. 1. Total aerobic count on (a) smoked pork loin and (b) frankfurter sausages stored in (●) vacuum, (▲) N₂ or (■) CO₂ at 4°C. Arrows indicate the time of identification of isolates.

The initial microflora of the two products is shown in Table 5. The composition of the flora was heterogenous on both products. The dominant bacteria on smoked pork loin were in the order, *Flavobacterium* spp., *Arthrobacter* spp., *Pseudomonas* spp., coryneform bacteria and *Bacillus* spp. Yeast and moulds constituted 20% of the isolates from the smoked pork loin but were not found on the frankfurter sausages. The dominant organisms on the sausage were in the order *Bacillus* spp., coryneform bacteria, *Pseudomonas* spp., *Flavobacterium* spp. and *Microbacterium* spp.

At the end of the storage *Lactobacillus* spp. and two groups of unidentified bacteria dominated the flora of both products and in all gas atmospheres (Table 6). On the pork loin the unidentified bacteria belonged to group A and their number decreased in the order, vacuum > N₂ > CO₂. *Lactobacillus viridescens* was found in all gas atmospheres and the number decreased in the reverse order.

On the sausages the unidentified bacteria group A decreased in the same order as for the smoked pork, however, no isolates of this group were found in CO₂ packs. Strains belonging to the group B were found in vacuum packs. A high proportion of *Lact. viridescens* was consistent with the smoked pork loins found in N₂ and CO₂, while the amount in vacuum was significantly lower. Along with the *Lact. viridescens*

large amounts of homofermentative *Lactobacillus* spp. were also found. The proportion was especially large in CO₂ and N₂ atmospheres.

One strain of each unidentified bacteria group A, B and *Lactobacillus* group 1 (all strains isolated from vacuum-packed smoked pork loin) were, after subsequent subculturing in MRS and freezing, reclassified as a single *Lactobacillus* sp. The characteristics for the three strains were: anaerobic growth; acid but no gas from glucose; acid from galactose, mannose, sucrose, trehalose; no acid from amygdalin, cellobiose, lactose, maltose, melezitose, raffinose, xylose; hydrolysis of arginine.

No off odours were noted from any of the products at the end of storage. The sausage had no off flavour after 210 d in CO₂, but had a swollen appearance due to the dissolved CO₂.

Discussion

The shelf-life increased for both products in the order, vacuum < N₂ < CO₂. Other studies on meat products have also reported that N₂ and CO₂ have good effects on the storage life (Callow 1932; Ogilvy & Ayres 1951). The corresponding order of shelf-life for raw meat is N₂ < vacuum < CO₂ (Enfors *et al.* 1979; Blickstad *et al.* 1981; Erichsen & Molin 1981). Thus the higher the CO₂ concentration the longer the

Table 3. Microbial counts, headspace gas concentration, pH and drip of smoked pork loin stored in different gas atmospheres at 4°C

Initial gas atmos.	Storage time (d)	Total aerobic count (log n/g)	Total anaerobic count (log n/g)	Lactic acid bacteria (log n/g)	Yeast & mould (log n/g)	<i>Brochothrix thermosphacta</i> (log n/g)	Analysed* CO ₂ (%)	Analysed* O ₂ (%)	pH	Drip (w/w, %)
vac	0	2.5	1.1	<1.0	1.6	<1.0	nd	nd	5.9	nd
Vac	6	2.4	1.6	1.2	1.6	<1.0	nd	nd	6.0	5.1
Vac	20	3.4	3.4	4.6	1.7	<1.0	nd	nd	6.0	6.1
Vac	41	7.5	7.4	5.3	3.0	1.9	95	0.1	5.8	5.8
Vac	48	7.6	7.4	6.0	3.0	<1.6	86	0.6	5.8	5.2
CO ₂	6	2.0	1.5	<1.1	<1.0	<1.0	100	0.0	6.0	1.1
CO ₂	20	3.0	3.0	2.8	<1.0	<1.0	98	0.1	5.8	1.8
CO ₂	41	6.4	6.4	5.3	<1.2	1.9	100	0.0	5.8	2.2
CO ₂	48	6.9	6.9	5.5	<1.0	<1.0	97	0.2	5.9	0.8
CO ₂	112	7.4	7.5	6.6	<1.0	<1.0	92	0.4	6.0	0.4
N ₂	6	2.1	<1.0	<1.0	<1.1	<1.0	4.2	0.8	6.0	1.2
N ₂	20	5.3	5.3	<3.4	<1.4	<1.0	4.7	0.0	5.9	0.7
N ₂	34	6.4	7.5	3.8	<3.2	2.2	5.6	0.1	5.9	0.0
N ₂	48	7.2	7.3	6.0	1.9	3.1	7.8	0.1	5.9	1.5
N ₂	112	7.6	7.6	7.0	<1.8	<1.2	16.9	0.0	5.7	1.2

* At the time of opening.

n, Number of colonies.

nd, Not determined.

Table 4. Microbial counts, headspace gas concentrations, pH and drip of frankfurter sausages stored in different gas atmospheres at 4°C

Initial gas atmos.	Storage time (d)	Total anaerobic count (log n/g)	Total anaerobic count (log n/g)	Lactic acid bacteria (log n/g)	Analysed* CO ₂ (%)	Analysed* O ₂ (%)	pH	Drip (w/w, %)
—	0	1.7	0.5	<1.0	nd	nd	5.9	nd
Vac	6	2.3	1.3	1.6	nd	nd	5.9	1.3
Vac	20	2.0	1.9	<1.6	nd	nd	5.8	1.3
Vac	48	4.2	3.4	<1.0	12	0.4	5.8	1.8
Vac	62	3.6	3.1	1.2	18	0.0	5.8	1.6
Vac	98	9.0	6.8	6.7	21	0.0	5.4	0.1
CO ₂	6	2.0	1.5	<1.1	99	0.0	5.7	2.2
CO ₂	20	1.8	2.8	<1.2	98	0.1	5.6	1.3
CO ₂	48	2.6	2.7	2.2	98	0.0	6.0	1.2
CO ₂	62	1.8	1.5	1.3	99	0.0	5.6	0.7
CO ₂	140	2.4	2.3	1.7	100	0.0	5.6	0.4
CO ₂	210	1.9	1.6	1.3	nd	nd	5.6	0.0
N ₂	6	2.1	<1.0	<1.0	0.5	0.5	6.0	0.1
N ₂	20	1.5	1.8	<1.3	0.4	0.4	5.8	0.1
N ₂	41	1.9	1.3	<1.0	nd	nd	5.8	0.1
N ₂	62	3.2	3.1	3.0	0.3	0.0	5.8	0.0
N ₂	98	4.8	4.1	4.7	0.4	0.0	5.8	0.0
N ₂	140	4.8	4.6	4.5	0.2	0.0	5.9	0.0

* At the time of opening.

n, Number of colonies.

nd, Not determined.

Table 5. The microbial flora of smoked pork loin and frankfurter sausages taken directly from the processing plant

Organisms	Distribution (%)	
	Smoked pork loin	Frankfurter sausage
<i>Acinetobacter calcoaceticus</i>	1	—
<i>Flavobacterium</i> spp.	22	8
<i>Moraxella osloensis</i>	—	1
<i>Moraxella phenon</i> 3*	2	—
<i>Moraxella phenon</i> 4*	1	—
<i>Pseudomonas</i> spp. (mesophiles)	—	11
<i>Pseudomonas</i> spp. (psychrotrophs)	11	—
<i>Bacillus brevis</i>	2	6
<i>Bacillus firmus</i>	—	26
<i>Bacillus megaterium</i>	5	1
<i>Bacillus pumilis</i>	2	—
<i>Bacillus subtilis</i>	—	1
<i>Arthrobacter</i> spp.	20	—
Coryneform bacteria	10	34
Unidentified bacteria group A	1	—
<i>Kurthia zopfii</i>	1	—
<i>Microbacterium</i> spp.	—	8
<i>Brochothrix thermosphacta</i>	—	1
<i>Listeria</i> sp.	1	—
<i>Micrococcus</i> sp.	—	1
<i>Staphylococcus aureus</i>	—	1
Yeast	20	—
Total aerobic count (log n/g)	2.5	1.7

* Thornley (1967).

Table 6. The microbial flora of smoked pork loin and frankfurter sausages after storage in different gas atmospheres at 4°C

Organisms	Distribution (%)					
	Smoked pork loin			Frankfurter sausage		
	Vacuum 48 d	CO ₂ 48 d	N ₂ 48 d	Vacuum 98 d	CO ₂ 140 d	N ₂ 140 d
<i>Lactobacillus coryne-</i> <i>formis</i> ssp. <i>torquens</i>	—	—	—	—	8	5
<i>Lactobacillus delbrueckii</i>	—	—	2	—	—	—
<i>Lactobacillus plantarum</i>	—	—	2	—	—	—
<i>Lactobacillus</i> sp. group 1*	2	—	—	8	58	45
<i>Lactobacillus</i> sp. group 2*	—	—	—	25	—	—
<i>Lactobacillus</i> spp.*	10	2	13	—	8	8
<i>Lactobacillus viridescens</i>	40	72	50	5	22	35
<i>Leuconostoc dextranicum</i>	—	2	—	—	—	—
<i>Erysipelothrix</i> -like	5	—	2	—	5	—
<i>Staphylococcus</i> sp.	—	—	—	2	—	—
Unidentified bacteria group A	41	23	30	30	—	8
Unidentified bacteria group B	2	—	—	30	—	—
Total aerobic count (log n/g)	7.6	6.9	7.2	9.0	2.4	4.8

* Unidentifiable homofermentative spp.

shelf-life of raw meat. The situation on processed meat seems to be more complex, i.e. the shelf-life does not increase together with the increase in CO₂ concentration. Obviously, there must be at least one additional factor controlling the microbiological development. This is also indicated by the atypical growth curves found for the frankfurter sausages stored in N₂ or CO₂ where the growth was retarded at low total aerobic count (Fig. 1b). According to Enfors & Molin (1980) and Blickstad *et al.* (1981), pure CO₂ of 1 atm should not alone be able to stop the growth of lactic acid bacteria.

Another environmental parameter that may be involved is the water activity. This is indicated by the various amounts of drip found in the packages. The retardation of the growth in N₂ and CO₂ (Fig. 1) seemed to be related to a concomitant decrease in the drip. Thus, it could be suggested that the microbiological development on the products studied is controlled chiefly in part by the CO₂ concentration and in part by the water activity.

The hypothesis that the CO₂ concentration together with the water activity controls the microbiological development does not fully explain why the shelf-life for the smoked pork loin was shorter than for the sausage. Neither is the

difference in shelf-life explained by variations in salt concentration, since both products were similar in this respect. However, the difference in the heat processing of the products may offer an explanation.

The initial flora on the smoked pork loin resembled the flora found on fresh pork loin (Enfors *et al.* 1979; Blickstad *et al.* 1981). However the genera *Arthrobacter* and *Bacillus* were not found on the fresh unheated loin.

The initial flora on the sausage had a higher proportion of *Bacillus* than the smoked pork loin. This may be due to the heat treatment reaching a higher internal temperature. The high numbers of *Bacillus* is in accordance with the observations of Chyr *et al.* (1980) who found an increasing proportion of *Bacillus* with increasing processing temperature on liver sausage.

In the present study *Bacillus megaterium* was the only psychrotrophic *Bacillus* found. The practical significance of psychrotrophic *Bacillus* spp. for shelf-life seems small, as no indication of the growth of these organisms was noticed. However, *Bacillus* spp. have been associated with the spoilage of semi-preserved canned sausages (Mitrica & Granum 1979).

In spite of the fact that no *Lactobacillus* spp.

were detected on the fresh product, this genus played a key role during storage. Even on the CO₂-stored sausages, where practically no growth occurred according to the total aerobic count, there was a shift in the flora towards a dominance of *Lactobacillus* spp. (95% of the flora; Table 6). The importance of *Lactobacillus* spp. in connection with the microbiological shelf-life of vacuum-packed processed meat has been shown by others (Allen & Foster 1960; Kempton & Bobier 1970).

It is an accepted fact that many of the *Lactobacillus* spp. isolated from meat and meat products cannot be identified and grouped into the existing taxonomic system. Terms such as 'unidentified' or 'atypical streptobacteria' are frequently used (Reuter 1970; Mol *et al.* 1971; Kitchell & Shaw 1975; Sharpe 1979). In the present study a majority of the 'unidentified' *Lactobacillus* were homofermentative and could be divided into two large groups. Organisms belonging to group 1 were found in greatest numbers in environments giving a low final total aerobic count. *Lactobacillus* group 2 was only found on vacuum-packed sausage and showed some resemblance to the streptobacteria isolated by Mol *et al.* (1971) and Reuter (1970). However one important criterion of Reuter's atypical streptobacteria, namely the inability to grow at pH 3.9, was not fulfilled.

Mol *et al.* (1971) suggested that a cooked product with a low salt concentration stored in an anaerobic or semi-anaerobic environment would favour the growth of the 'atypical streptobacteria', while Kitchell & Shaw (1975) suggested that a low storage temperature together with a CO₂ atmosphere were favourable. The results in the present study do not contradict these suggestions.

Lactobacillus viridescens was found in large numbers, especially on the smoked pork loin. This organism is frequently found in variable amounts on cured meat products (Sharpe 1962; Mol *et al.* 1971; Reuter 1975) and is, due to its heterofermentative behaviour, known to have an undesirable influence on product quality. The amount of *Lact. viridescens* developing in the present study was apparently independent of the package atmosphere (Table 6). There are minor variations, but it may be unwise to draw any conclusions due to the fact that the composition of a *Lactobacillus* flora could be changing with storage time (Collins-Thompson &

Lopez 1980) and, for example, the proportion of *Lact. viridescens* may decrease with storage time.

Lactobacillus viridescens is known to cause greening of meat products by producing hydrogen peroxide. The hydrogen peroxide reacts with the meat pigments and a green colour develops after exposure to air (Jay 1978). In the present study the products were not exposed to air after microbiological sampling and hence it was not noticed if any greening occurred.

Other studies on vacuum-packed meat products have demonstrated a total dominance of lactic acid bacteria, mainly *Lactobacillus* spp. (Allen & Foster 1960; Kempton & Bobier 1970). In the present study *Lactobacillus* constituted 38–96% of the final microflora (Table 6). When a low proportion of *Lactobacillus* was found, the count of lactic acid bacteria on acetate agar was also significantly lower than the total aerobic count. In these cases (smoked pork loin: vacuum, N₂, CO₂; sausages: vacuum—Tables 3 and 4) significant numbers of unidentified bacteria were found (Table 6). This indicates that the unidentified bacteria were unable to grow on acetate agar.

Most organisms not identified as *Lactobacillus* spp. could not be assigned to any known genera (8–60% of the final microflora; Table 6).

The unidentified strains belonging to group A could not be identified as *Lactobacillus* spp. since they did not produce acid from glucose, neither were they likely to belong to the coryneforms since they were catalase-negative (Buchanan & Gibbons 1974). However, Gram positive non-sporing rods are frequently classified as coryneforms in the literature (Jones 1978).

In a study on vacuum-packed bacon Kitchell & Shaw (1975) demonstrated that 80% of their isolates of *Lactobacillus* were unable to grow on acetate agar but after subsequent subculturing they were adapted to do so. In the present study it was found that some strains, primarily classified as 'unidentifiable', acquired other properties after subsequent subculturing on laboratory media, i.e. they could be reclassified as a *Lactobacillus* sp. This indicates that groups A and B of 'unidentifiable' strains may be *Lactobacillus* spp. which first needed to be accustomed to laboratory media before they behaved as *Lactobacillus* spp. (acid from glucose; anaerobic growth). It is debateable whether or not a wild-

type strain should be subcultured for a couple of times in order to make its biochemical activities uniform before the 'taxonomical' identification, i.e. should the wild-type strain be domesticated before identification? From a standard classification point of view the answer is probably: 'Yes, it should'. However, from a more ecological standpoint the answer may be different. Here the physiological and biochemical profile of the organism within a specific environment may be of greater interest than the more exact taxonomical position of the organism. Nevertheless, whatever the designation of the unidentified bacteria of groups A and B of the present study should be, they seem to have an ecological significance for the shelf-life of cured meat.

In conclusion, this study shows that frankfurter sausage can achieve an extremely good microbiological shelf-life when stored in CO₂ at low temperatures. A closer understanding of the factors involved will enable a better microbiological control of the shelf-life of refrigerated processed meat.

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Growth and Lactic Acid Production of *Pediococcus pentosaceus* at Different Gas Environments, Temperatures, pH Values and Nitrite Concentrations

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Summary. In order to obtain a better understanding of the behaviour of *Pediococcus pentosaceus* in food products as well as to facilitate the designing of industrial production processes for the organism, the growth and lactic acid production of *Pediococcus pentosaceus* in a complex glucose medium was followed in batch cultures at different gas environments (CO_2 , air, N_2 and static cultures without gasflow), temperatures ($10\text{--}50^\circ\text{C}$), pH (4.3–7.3) and nitrite concentrations (0–700 ppm). Optimal growth was obtained in CO_2 at 40°C and pH 6.3 and resulted in a maximum specific growth rate ($\mu_{\max}$) of 1.27 h^{-1} . In static culture at 40°C and pH 6.3 the $\mu_{\max}$ was 1.21 h^{-1} . The $\mu_{\max}$ was, compared with static culture, reduced in air (12%) and nitrogen (26%). At 10°C the $\mu_{\max}$ was reduced by 99% and at 50°C by 88%. The reduction at pH 4.3 and 7.3 was 65% and 57%, respectively. Nitrite did not affect the $\mu_{\max}$ at any pH but increased the lag phase at pH 4.3 by a factor of 12. The lactic acid production was linked to the growth. The total amount of lactic acid produced was the same in all the tested gases and nitrite concentrations and also within the wide temperature range ($15\text{--}45^\circ\text{C}$) and pH range (5.3–7.3). Mainly L(+)-lactic acid was produced during the exponential growth phase, but after this growth declined about 30% of the L(+)-lactic acid was converted to D(–)-lactic acid. The lactic acid product yield and the cellmass yield were both affected by the temperature but not by the pH.

Paddock (1940). *Pediococcus cerevisiae* (now *P. acidilactici* or *P. pentosaceus*; Anon 1979) has since been proved to be a suitable starter organism for meat products (Niven et al. 1959; Everson et al. 1970) and is now commercially available in many countries.

Pediococcus spp. contribute also to product development in other types of fermented food products such as sauerkraut and pickles (Jay 1978).

Pediococcus spp. are known to have an inhibitory effect on several spoilage organisms and pathogens (Gilliland and Speck 1975; Raccach et al. 1979) and have been demonstrated to be useful as a shelf life prolonging factor of, for example, poultry meat (Raccach et al. 1979).

Lactic acid bacteria have proved to be useful in bacon manufacturing by reducing the development of nitrosamines (Goodfellow 1979). Not all lactic acid bacteria are, however, suitable since nitrite may inhibit lactic acid bacteria (Shaw et al. 1978; Puolanne 1978).

Consequently, *Pediococcus* spp. have many applications in the food industry. In spite of this, few data of the growth kinetic and the physiology of this genus have been reported in the literature.

The intention of the present investigation has been to study growth and lactic acid formation of *Pediococcus pentosaceus* at different gas environments, temperatures, pH values and nitrite concentrations. Such information should contribute (i) to a better understanding of this organisms behaviour in food products and (ii) to designing industrial processes for the production of *Pediococcus*-starters.

Introduction

The use of lactic acid bacteria as a starter culture in fermented meat products was first described by Jensen and

Materials and Methods

The test organism *Pediococcus pentosaceus* was characterized by the following: growth at 12°C and 45°C but not at 50°C ; growth in 8% NaCl but not in 15% NaCl; no production of catalase; no production of CO_2 from glucose; acid formation from

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arabinose, sucrose and trehalose but not from maltose, sorbitol and starch; hydrolysis of arginine and aesculin. Stock cultures were stored frozen (-40°C) in Litmus milk (Difco) supplemented with 0.5% (w/v) glucose.

The growth medium (YTG) had the following composition (g l^{-1}): glucose 32.0; Yeast extract (Difco), 24.0; Tryptone (Difco), 5.0; CH_3COONa , 1.0; KH_2PO_4 , 0.70; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.22; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.06. The glucose was autoclaved separately and added aseptically. The medium was adjusted to the desired pH with HCl or NaOH. The bacteria were subcultured before inoculation in the following way: (i) the frozen cultures were incubated in the Litmus milk (30°C , 24 h); (ii) 2 ml was transferred into 200 ml YTG and incubated (30°C , 15.5 h); (iii) 10 ml was transferred into 200 ml YTG and incubated (at the temperature used during cultivation, 110 min); (iv) these 200 ml were used for inoculating. The cell concentration in the fermentor after inoculation was about $3 \cdot 10^6$ cells per ml.

The cultures were performed in a 10 l fermentor (Chemoform AB, Hagersten Sweden) fitted with equipment to control temperature (YSI 73A, Yellow Springs Instrument Co., Yellow Springs, Ohio) and pH (Radiometer PHM 62 + TTT 60 titrator, Copenhagen, Denmark). The pH was held at the desired value by titration with NaOH (3.5 M). The feeding of NaOH was continuously monitored by a Dose Monitore[®] (Servo Chem AB, Vallingby, Sweden) and was recorded (KSQ 536/63, Jaquet Ltd., Basel, Switzerland). The gases used, CO_2 and N_2 , (Alfax, Malmö, Sweden) had a purity of 99.998% and 99.999%, respectively. The gas flow was measured by a rotameter. The stirring speed was 300 rpm.

Batch cultures were performed at different gas atmospheres, temperatures, pH values and nitrite concentrations. Gas atmospheres applied were pure CO_2 , N_2 and air (pH 6.3, 40°C). The medium was sparged (421 h^{-1}) with gas 2 h or more before inoculation and throughout cultivation. A static gas atmosphere was used in all the other cultures, that is the media was sparged with N_2 (171 h^{-1}) 20 min before inoculation but not any further. Temperatures applied were 10, 15, 20, 25, 30, 35, 40, 45 and 50°C (pH 6.3, static culture). The pH applied were 4.3, 4.8, 5.3, 5.8, 6.3, 6.8 and 7.3 (40°C , static culture). The initial nitrite (NaNO_2) concentrations applied were 200, 500 and 700 ppm (pH 6.3, 40°C , static culture). Cultures with 200 ppm nitrite were also performed at pH 5.3 (40°C), pH 4.3 (40°C) or 30°C (pH 6.3).

Growth was followed by viable count ($\log_{10}$ cells ml^{-1}), dry weight (g l^{-1}) and absorbance at 620 nm (A_{620}). Bacterial counts were made by conventional pour plate technique on MRS agar (Oxoid). Plates were incubated at 37°C for 3 days. Dry weight was determined by filtering 5 ml bacterial suspension through a 0.45μ membranefilter (Millipore[®]) and washing twice with equal volumes of distilled water. The filter was dried at 105°C for 1 h before weighing.

Lactic acid production was followed continuously by the feeding of NaOH to the fermentor. The amount of lactic acid produced was determined either from the titration with NaOH or enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, G.D.R.). The enzymatic method gave the amounts of L(+) and D(−)-lactate produced. Samples withdrawn for enzymatic analysis were centrifuged (2500 rpm, 20 min) and the supernatant liquid was stored at -40°C until determination.

The amount of glucose consumed was determined enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, G.D.R.). The samples were centrifuged and stored in the same way as for lactic acid determination.

Nitrite was determined by the method of Adriaanse and Robbers (1969). Some of the nitrite added to the medium reacted at once, and the amounts of NO_2^- analyzed immediately after the addition of 200 ppm was 162 ppm at pH 6.3, 93 ppm at pH 5.3 and 21 ppm at pH 4.3.

The reported maximum specific growth rates ($\mu_{\text{max}} = \frac{dx}{dt} \cdot \frac{1}{x}$; where x is the cellmass and t the growth time) were based on measurements of the absorbance. The correlation between dry weight and A_{620} was high (> 0.94).

The relative reduction in μ_{max} was calculated as $(r_s - r) 100/r_s$; where r_s is the μ_{max} obtained in static culture at 40°C and pH 6.3, while r is the μ_{max} for which the relative reduction is to be calculated.

The specific production rate of lactic acid ($q_p = \frac{dP}{dt} \cdot \frac{1}{x}$; where P is the produced lactic acid (mmol l^{-1}), calculated from the titration with NaOH, t is the growth time (h) and x is the cellmass (g l^{-1})).

The overall product yield (Y_p = lactic acid produced per glucose consumed, g g^{-1}) and the overall growth yield (Y_x = biomass produced per glucose consumed, g g^{-1}) were calculated.

Results

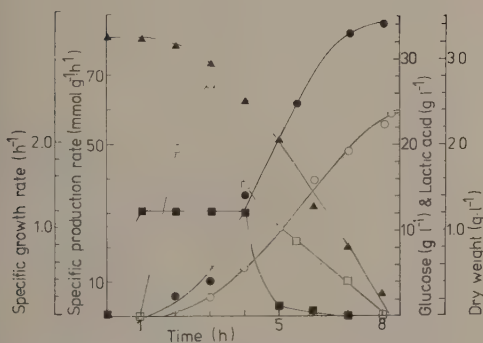
The maximum specific growth rates of *Pediococcus pentosaceus* at different gas environments, temperatures and pH values are given in Table 1. Highest μ_{max} values were obtained in CO_2 (1.27 h^{-1}) and static culture (1.21 h^{-1}) at 40°C and pH 6.3. In air and N_2 the μ_{max} was reduced, compared to static culture, by 12% and 26%, respectively. The highest viable count was found in CO_2 ($9.8 \log$ cells ml^{-1} corresponding to 3.5 g l^{-1} dry weight), while a slightly lower count was found in the other gas environments. The stationary phase, with respect to the viable count, was prolonged when cultures were sparged with gas. After the static cultures had reached their maximum population, the viable count decreased by 0.7 log cells ml^{-1} during a 12 h period. No decrease was found in cultures sparged with gas. A viable count above 9 log cells ml^{-1} was attained in all environments tested except at 10°C , 50°C and pH 7.3. None of the tested nitrite concentrations affected the μ_{max} or the terminal viable count.

The amount of lactic acid produced was affected by the environment (Table 1). At 10°C , 50°C or pH 4.3 lower amounts of lactic acid was produced. Nitrite had no effect on the amount of lactic acid produced. However, the lag phase, with respect to lactic acid production and cell growth, was prolonged in the presence of 200 ppm nitrite at pH 4.3, i.e. the lag phase increased from 2 h to 25 h.

The link between growth and lactic acid production in a static culture at pH 6.3 and 40°C is shown in Fig. 1. The μ_{max} was reached earlier than the maximum specific production rate of lactic acid (q_p). The μ_{max} was reached after 1.0 h and decreased 3.0 h later while q_p reached its highest level after 3.0 h. The decrease in μ_{max} started when 6 g l^{-1} lactic acid had been produced. The increase in viable count stopped when the lactic acid concentration reached 20 g l^{-1} while lactic acid production continued until the glucose was depleted.

Table 1. Growth and lactic acid production of *P. pentosaceus* at different gas environments, temperatures and pH

Gas atmosphere	temperature (°C)	pH	$\mu_{\max}$ (h ⁻¹)	relative reduction of $\mu_{\max}$ (%)	maximal viable count (log ₁₀ cells ml ⁻¹)	produced lactic acid (g l ⁻¹)
static	40	6.3	1.21	0	9.6	25
CO ₂	40	6.3	1.27	-5*	9.8	26
air	40	6.3	1.06	12	9.6	25
N ₂	40	6.3	0.89	26	9.5	23
static	10	6.3	0.005	99	7.3	0.5
static	15	6.3	0.10	92	9.0	24
static	20	6.3	0.22	82	9.4	23
static	30	6.3	0.62	49	9.3	25
static	35	6.3	0.83	31	9.4	23
static	45	6.3	1.09	10	9.1	24
static	50	6.3	0.14	88	6.7	2
static	40	4.3	0.42	65	9.3	15
static	40	5.3	0.95	21	9.4	25
static	40	5.8	1.03	15	9.3	24
static	40	6.8	0.99	18	9.2	25
static	40	7.3	0.52	57	8.8	24

* increase of $\mu_{\max}$ **Fig. 1.** *P. pentosaceus* growing on a complex glucose medium in a static culture at 40 °C and pH 6.3; (■) specific growth rate; (□) specific lactic acid production rate; (▲) glucose concentration; (○) lactic acid concentration; (●) dry weight

The product yields (Y_p) and the growth yields (Y_x) at different pH and temperatures are shown in Figure 2. Neither of the yields were significantly influenced by pH ($Y_p = 0.8 \text{ g g}^{-1}$; $Y_x = 0.12 \text{ g g}^{-1}$), but both were affected by the temperature. Y_p was reduced by 50% at 10 °C, and Y_x varied irregularly within the range 0.09–0.12.

The yields were slightly affected by the gas environment. In N₂ a somewhat lower Y_p (0.72 g g⁻¹) was found. The growth yields was slightly lower (0.10 g g⁻¹) in the three gas environments tested compared to static culture.

The nitrite levels tested did not significantly affect Y_p . The growth yield was slightly lower with 700 ppm nitrite at 40 °C (0.09 g g⁻¹), 200 ppm at 30 °C (0.08 g g⁻¹) and 200 ppm at pH 5.3 (0.06 g g⁻¹).

The relation between the produced amount of L(+) and D(-)-lactic acid was determined in static culture (40 °C, pH 6.3). The L-form dominated (82%) at the point when the glucose was depleted. About 14 h later 7 g l⁻¹ of L-lactic acid had been changed into D-lactic acid, i.e. 54% of the lactic acid was in L-form. The total amount of lactic acid was unchanged.

Discussion

The highest $\mu_{\max}$ obtained for *P. pentosaceus* was 1.27 h⁻¹. This is higher than growth rates reported for other lactic acid bacteria such as *Streptococcus cremoris* (0.85 h⁻¹, Lee and Collins 1976; 0.61 h⁻¹, Cogan et al. 1971), *Streptococcus lactis* (0.94 h⁻¹, Lee and Collins 1976), *Lactobacillus casei* (0.60 h⁻¹, de Vries et al. 1970) or *Leuconostoc cremoris* (0.28 h⁻¹, Cooper and Collins 1978). An exception is 1.34 h⁻¹ reported for *Streptococcus lactis* (Cogan et al. 1971).

Sparging the culture with air or N₂ decreased the $\mu_{\max}$ in comparison to CO₂ or static culture. This may be caused by flushing out endogenously produced CO₂ with the applied gas flow. CO₂ has been reported necessary for the growth of *Lactobacillus arabinosus* (Lardy et al. 1949),

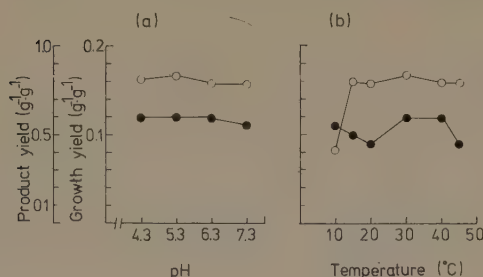


Fig. 2. a and b. (○) product yield and (●) growth yield of *P. pentosaceus* on glucose at different a pH and b temperatures

Streptococcus lactis (Cogan et al. 1971) and *S. sanguis* (Repaske et al. 1974).

No effort was made in the present study to determine the amount of nitrite needed for a total inhibition of *Pediococcus pentosaceus*. Instead the nitrite levels were chosen to correspond to those of practical interest in meat products. 25,000 ppm nitrite has been reported to totally inhibit the growth of *P. cerevisiae* at pH 6.6 (Castellani and Niven 1955). Other lactic acid bacteria seem to be more sensitive, e.g. *Lactobacillus casei* was inhibited by 8,000 ppm (Castellani and Niven 1955). However lower nitrite concentrations may also affect the growth. A level of 100 ppm nitrite has been reported sufficient to reduce the growth of *L. plantarum* (Puolanne 1978). The present study showed that *Pediococcus pentosaceus* tolerated nitrite concentrations up to 700 ppm without any decrease in growth rate. However, nitrite did have some effect at low pH where the lag phase was prolonged. The action of nitrite on *Staphylococcus aureus* has also been demonstrated to be dependent on pH (Castellani and Niven 1955).

The product yields in the present study were low (mean value 0.80 g g⁻¹) compared to homofermentative bacteria in general. According to Garvie (1980) the product yield should be at least 0.85 for homofermentative strains. However, Hanson and Tsao (1972) reported values down to 0.74 g g⁻¹ for *Lactobacillus delbrueckii*. Thus, the values found in the present study are not extremely low.

At 10 °C the product yield decreased to 0.41 g g⁻¹ (Fig. 2). This may be due to a switch from homofermentative to heterofermentative metabolism. Such a switch has been demonstrated for *L. bulgaricus* at alkaline pH (Rhee and Pack 1980).

The test organism in the present study produced mainly L-lactic acid during the exponential growth. Some of this was later converted to D-lactic acid and a racemic mixture of L- and D-form was found 14 h after substrate depletion. The fact that a *Pediococcus* sp. produces a racemic mixture of L- and D-form is in accordance with

the literature (Buchanan and Gibbons 1974). However, as the D-form was mainly formed after growth decline, and in view of the present literature data of how lactic acid bacteria are able to produce D-lactic acid (Garvie 1980, Gordon and Doelle 1975), the indication is that the *P. pentosaceus* strain possesses a racemase. No lactate racemase has been reported for any *Pediococcus* sp. (Garvie 1980). Nevertheless racemase has been isolated from different *Lactobacillus* spp. (Stetter and Kandler 1973).

In conclusion, it may be said that production of a starter culture based on the present strain of *Pediococcus pentosaceus*, in simple batch procedure, is most preferably performed at 40 °C, pH 6.3 and under a relatively high partial pressure of CO₂. Under these conditions 6 · 10⁹ cell forming units per ml growth medium can be produced within a 7 h growth cycle. Moreover, the study indicated that *P. pentosaceus* is able to grow and produce lactic acid even within a varied environment of relatively wide limits. The tolerance against nitrite and air must be regarded as especially valuable features for an organism to be used as a starter in meat products.

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Growth and End Product Formation of Two Psychrotrophic *Lactobacillus* spp. and *Brochothrix thermosphacta* ATCC 11509^T at Different pH Values and Temperatures

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Lactobacillus viridescens, *Lactobacillus* sp. strain 173 (homofermentative), and *Brochothrix thermosphacta* ATCC 11509^T were studied at different pH values and temperatures in aerobic and anaerobic batch cultures. The growth rates were higher in aerobic than in anaerobic cultures. *L. viridescens* grew faster at pH 5.8 than at pH 6.3, whereas the opposite was true for *B. thermosphacta*. *Lactobacillus* sp. strain 173 was inhibited in air or at 8°C in anaerobic culture. *B. thermosphacta* did not grow in anaerobic culture at pH 5.3. The following variations in growth yields were found in the different environments studied: *Lactobacillus* sp. strain 173, 23 to 25 g (dry weight) per mol of glucose consumed; *L. viridescens*, 11 to 23 g/mol; *B. thermosphacta*, 16 to 38 g/mol. In air, *L. viridescens* produced D-lactic acid, ethanol, and acetic acid, whereas no acetic acid was produced anaerobically. Acetic acid and ethanol together constituted 41 to 48% of the total product yield irrespective of pH and temperature. *Lactobacillus* sp. strain 173 produced a racemic mixture of D- and L-lactic acid at pH 6.3, whereas the proportion of L-lactic acid was higher than that of D-lactic acid at pH 5.3. In air, product formation of *B. thermosphacta* varied from a domination of L-lactic acid to increasing yields of acetoin, acetic acid, 2,3-butanediol and isovaleric acid. The effect of pH and temperature on product formation was difficult to separate from the effect of O₂ availability in aerobic cultures. However, it was indicated that more 2,3-butanediol and less acetoin were produced with a decreasing temperature. In anaerobic cultures, L-lactic acid and small amounts of formic acid were produced by *B. thermosphacta*, irrespective of the temperature.

Bacterial growth on meat and meat products is affected by factors such as pH, temperature, and gas atmosphere. In different environments, different organisms will dominate, and to understand both how spoilage arises and how to prevent it, it is important to know in what way significant bacteria are affected.

Lactobacillus spp. and *Brochothrix thermosphacta* significantly influence the quality of meat and meat products. On vacuum- or gas-packed meat and meat products, *Lactobacillus* spp. often dominate the microflora (1, 2, 4, 9). *B. thermosphacta* has on several occasions been found together with *Lactobacillus* spp. (9, 13, 16).

The physiology of *B. thermosphacta* has been studied by several authors (6, 8, 10), whereas *Lactobacillus* spp. from meat or meat products have been given much less attention. This can perhaps be explained by the fact that it is at present impossible to identify the majority of the *Lactobacillus* spp. found on meat or meat products since their characteristics do not conform

with any described, approved species of *Lactobacillus* (4, 11). Thus, it is difficult to choose representative test strains. However, *Lactobacillus viridescens* has been isolated from meat (2) and meat products (4) and is associated with green discolorations (12).

The present work studies the influence of different pH values and temperatures on the growth of one homofermentative and one heterofermentative *Lactobacillus* sp. and the *B. thermosphacta* type strain.

MATERIALS AND METHODS

Organisms. The test organisms used were *L. viridescens* SMRICC174, *Lactobacillus* sp. strain SMRICC173, and *B. thermosphacta* ATCC 11509^T. *L. viridescens* 174 was characterized by the following: acid formation from cellobiose, fructose, glucose, maltose, mannose, ribose, and sucrose but not from amygdalin, arabinose, galactose, lactose, mannitol, melezitose, raffinose, rhamnose, trehalose, and xylose; no hydrolysis of arginine; and gas production from glucose. *Lactobacillus* sp. strain 173 was charac-

terized by the following: acid formation from fructose, galactose, glucose, mannose, ribose, sucrose, and trehalose but not from amygdalin, arabinose, cellobiose, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, and xylose; hydrolysis of arginine; and no gas production from glucose.

Medium. The growth medium (YTGT) had the following composition (g/liter): yeast extract (Difco Laboratories, Detroit, Mich.), 24.0; glucose, 20.0; tryptone (Difco), 5.0; sodium acetate, 1.0; KH_2PO_4 , 0.70; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.22; silicone antifoaming agent (30% wt/wt; BDH Chemicals Ltd., Poole, England), 0.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.06; thiamine-hydrochloride, 0.001. The glucose was autoclaved separately.

Growth experiments. The cultures were performed batchwise in a 1-liter fermentor at controlled pH and temperature. The flow rate of the gas was 17 liters/h, and the stirring rate was 400 rpm. The medium was sparged with the applied gas for 20 min before inoculation and then throughout the cultivation. The inoculum was subcultured twice before inoculating the fermentor to a concentration of 4×10^6 cells per ml.

Batch cultures were performed aerobically or anaerobically (5% CO_2 plus 95% N_2) at different pH values (5.3, 5.8, and 6.3) and temperatures (8°, 15°, and 25°C). The initial pH was adjusted with 2 M HCl. A constant pH during the growth was maintained by automatic titration (Radiometer PHM62 + TTT60 titrator, Copenhagen, Denmark) with 1 M NaOH.

Analysis. Growth was followed by viable count (pour plate technique with APT agar [BBL Microbiology Systems, Cockeysville, Md.] and incubation at 25°C for 3 days), determination of dry weight (3), and absorbance at 620 nm. Samples were taken out once every half hour during the daytime.

Samples (two of 15 ml each) withdrawn from the fermentor were centrifuged ($2,500 \times g$ for 20 min) at room temperature, and the supernatant liquid was stored at -40°C until determination was carried out. The concentrations of acetic acid, ethanol, formic acid, glucose, D-lactic acid, and L-lactic acid were determined enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, German Federal Republic). The concentrations of acetoin, isobutyric acid, isovaleric acid, and 2,3-butanediol were, after ether extraction (8), determined on a gas chromatograph (Varian 400; Varian Associates, Inc., Palo Alto, Calif.). A 3-ft (ca. 91.44-cm) glass column (Chromosorb WAW, 80-100 mesh and 10% DEGA; OVSC, Marietta, Ohio) and a flame ionization detector were used. The flow rate in the column was 40 ml of N_2 per min, and the flow rate in the detector was 30 ml of H_2 per min plus 190 ml of air per min. The temperature in the column was raised from 90 to 140°C (6°C/min). The injector temperature was 150°C, and the detector temperature was 200°C (the method was described in a personal communication by C. Hibbard, Meat Research Institute, Langford, United Kingdom). The presence of hydrogen peroxide was detected with Perid strips (detection limit 5 mg/liter; Boehringer Mannheim).

Calculations. The calculation of the maximum specific growth rate (μ_{max}) was done according to Pirt (14) and by using the absorbance at 620 nm as a measure of cell mass. The overall growth yield (Y_x = gram [dry weight] produced per mole of glucose consumed) and

the overall product yield (Y_p = mole of product formed per mole of glucose consumed) were calculated.

RESULTS

Effect of pH. The highest specific growth rate (μ_{max}) of *L. viridescens* was found at pH 5.8 in aerobic culture (0.50 h^{-1} , Table 1). The μ_{max} values obtained in aerobic cultures were higher than those in corresponding anaerobic cultures. The growth yield (Y_x) was about 22 g/mol with minor variations at the different pH values applied, except at pH 5.3 in anaerobic culture, where a much lower cell mass was detected (Table 1). *L. viridescens* produced the same type of end products irrespective of pH but with slightly varying yields (Table 1). D-Lactic acid dominated the end products. Ethanol was produced aerobically as well as anaerobically, whereas acetic acid was found only in aerobic culture. The heterofermentative end products (other than lactate) constituted 41 to 48% of the total product yield. Hydrogen peroxide was produced in aerobic cultures.

Lactobacillus sp. strain 173 was inhibited in air at a low final dry weight (inhibited culture, 0.4 g/liter; noninhibited culture, 2.6 to 3.0 g/liter). The μ_{max} was higher at pH 6.3 than at pH 5.3 (Table 1). The Y_x was about 24 g/mol (Table 1). *Lactobacillus* sp. strain 173 produced a racemic mixture of lactic acid at pH 6.3, whereas the proportion of L-lactic acid was slightly higher at pH 5.3 (Table 1). The highest μ_{max} of *B. thermosphacta* was found at pH 6.3 in air (0.49 h^{-1} , Table 1). The μ_{max} values obtained in aerobic cultures were higher than those in the corresponding anaerobic cultures. *B. thermosphacta* did not grow at pH 5.3 in anaerobic culture. At pH 5.3, the aerobic growth was terminated before all glucose was consumed, and the μ_{max} was 40% lower than that at pH 6.3. The Y_x decreased from 27 to 16 g/mol when the pH was decreased from 6.3 to 5.3 in aerobic culture (Table 1). The product formation of *B. thermosphacta* in air varied with the pH (Table 1). The yield of L-lactic acid decreased from 1.4 mol/mol at pH 6.3 to 0.8 mol/mol at pH 5.3. In addition to L-lactic acid, small yields of ethanol, acetic acid, acetoin, 2,3-butanediol, isovaleric acid, isobutyric acid, and formic acid were also found.

Effect of temperature. The μ_{max} values of *L. viridescens* were higher in aerobic cultures than in corresponding anaerobic cultures (Table 2). The growth yield varied between 18 and 22 g/mol, and the lowest yield was found at 8°C in anaerobic culture (Table 2). *L. viridescens* produced D-lactic acid as a dominating end product irrespective of temperature (Table 2). The heterofermentative end products constituted 41 to 48% of the total product yield. *Lactobacillus* sp.

TABLE 1. Growth and end product formation in aerobic and anaerobic cultures of *L. viridescens* SMRICC 174, *Lactobacillus* sp. strain SMRICC 173, and *B. thermosphacta* ATCC 11509[†] at different pH values^a

Organism	Gas atmosphere	pH	Maximum specific growth rate (h ⁻¹)	Growth yield (g dry wt) per mol of glucose consumed)	Product yield (mol of product per mol of glucose consumed) ^a								
					D-Lactic acid	L-Lactic acid	Ethanol	Acetic acid	Acetoin	2,3-Butanediol	Isovaleric acid	Isobutyric acid	Formic acid
<i>L. viridescens</i>	Aerobic	6.3 ^c	0.46	23	0.88	0	0.58	0.24	0	0	0	0	0
	Aerobic	5.8 ^c	0.50	22	0.96	0	0.60	0.24	0	0	0	0	0
	Aerobic	5.3 ^c	0.35	20	1.01	0	0.70	0.15	0	0	0	0	0
	Anaerobic	6.3 ^c	0.39	23	1.13	0	0.86	0	0	0	0	0	0
	Anaerobic	5.3	0.30	11	1.01	0	0.70	0	NA	NA	NA	NA	0
<i>Lactobacillus</i> sp. strain 173	Aerobic	6.3	Inhibited growth										
	Anaerobic	6.3	0.37	23	0.88	0.86	0	0	0	0	0	0	0
	Anaerobic	5.3	0.24	25	0.76	0.98	0	NA	NA	NA	NA	NA	NA
<i>B. thermosphacta</i>	Aerobic	6.3 ^c	0.49	27	0	1.42	0.08	0.12	tr	0.04	tr	tr	0.08
	Aerobic	5.8	0.45	20	0	1.06	0.04	0.15	0.02	0.06	tr	tr	0.01
	Aerobic	5.3 ^{c,d}	0.20	16	0	0.76	0	0.24	0.16	0.06	tr	tr	0
	Anaerobic	6.3 ^c	0.17	22	0	1.52	0.12	0	0	0	tr	tr	0.08
	Anaerobic	5.3 ^c	No growth										

^a Cultures were performed at 25°C in a complex medium with 2% glucose.

^b 0, Not detected; NA, not analyzed; tr, trace amounts below 0.01 mol of product per mol of glucose consumed.

^c Mean values from two cultures are reported.

^d Only 13.2 g of glucose per liter consumed.

TABLE 2. Growth and end product formation in aerobic and anaerobic cultures of *L. viridescens* SMRICC 174, *Lactobacillus* sp. strain SMRICC 173, and *B. thermosphacta* ATCC 11509^T at different temperatures^a

Organism	Gas atmosphere	Temp (°C)	Maximum specific growth rate (h ⁻¹)	Growth yield (g [dry wt] per mol of glucose consumed)	Product yield (mol of product per mol of glucose consumed) ^b							
					D-Lactic acid	Ethanol	Acetic acid	Acetoin	2,3-Bu-tanediol	Isovaleric acid	Isobutyric acid	Formic acid
<i>L. viridescens</i>	Aerobic	25 ^c	0.46	23	0.88	0	0.58	0.24	0	0	0	0
	Aerobic	15 ^c	0.20	22	0.96	0	0.53	0.22	0	0	0	0
	Aerobic	8	0.09	23	0	0	0.52	0.17	0	0	0	0
	Anaerobic	25	0.39	23	1.13	0	0.86	0	0	0	0	0
	Anaerobic	8	0.06	18	1.05	0	0.80	0	NA	NA	NA	0
<i>Lactobacillus</i> sp. strain 173	Aerobic	25	Inhibited growth									
	Anaerobic	25	0.37	23	0.88	0.86	0	0	0	0	0	0
	Anaerobic	8	Inhibited growth									
<i>B. thermosphacta</i>	Aerobic	25 ^c	0.49	27	0	1.42	0.08	0.12	tr	0.04	tr	0.08
	Aerobic	15 ^c	0.21	31	0	1.17	0.04	0.07	tr	0.09	tr	0.06
	Aerobic	8 ^c	0.08	38	0	0.04	0	0.32	0.39	0.02	tr	tr
	Anaerobic	25	0.17	22	0	1.52	0.12	0	0	tr	tr	0.08
	Anaerobic	8	0.05	20	NA	1.51	0.05	0	0	tr	0	tr

^a Cultures were performed at pH 6.3 in a complex medium with 2% glucose.

^b 0, Not detected; NA, not analyzed; tr, trace amounts below 0.01 mol of product per mol of glucose consumed.

^c Mean values from two cultures are reported.

strain 173 was inhibited at 8°C at a low final dry weight.

In aerobic cultures, *B. thermosphacta* grew at $\mu_{\max}$ values higher than those in the corresponding anaerobic cultures (Table 2). The Y_x varied between 27 and 38 g/mol in aerobic cultures, and an increase was found with decreasing temperature (Table 2). In anaerobic culture, the Y_x was about 21 g/mol and was unaffected by the temperature. In aerobic culture, the product yield of L-lactic acid decreased from 1.42 mol/mol at 25°C to 0.04 mol/mol at 8°C (Table 2). At 8°C in aerobic culture, acetoin dominated the end products together with acetic acid and 2,3-butanediol. In anaerobic culture, L-lactic acid dominated the end products even at 8°C, and no acetoin, acetic acid, or 2,3-butanediol was found.

DISCUSSION

The two *Lactobacillus* strains used in this study were isolated from spoiled smoked pork and spoiled pork. Homofermentative *Lactobacillus* spp. are frequently isolated from meat or meat products (2, 4, 9). *Lactobacillus* sp. strain 173 belonged to the *Lactobacillus* group 1 of Blickstad and Molin (4), representing 2 to 58% of the spoilage flora of cured meat products. *L. viridescens* is found on meat (2) and meat products (4) and is associated with the greening of meat products (12). *Lactobacillus* sp. strain 173 did not meet the description of any approved species of lactobacilli. Phenotypically, this strain can at best be placed somewhere between the type species of lactobacilli, *Lactobacillus delbrueckii* and *Lactobacillus mali*, from which it differs in the following characteristics: ribose, trehalose, and type of lactic acid (*L. delbrueckii*; 5); arginine, ribose, and rhamnose (*L. mali*; 7). Two features of the present strain of *L. viridescens* did not agree with the general description of *L. viridescens* (5), namely, fermentation of cellobiose and production of D-lactic acid instead of DL-lactic acid.

Lactobacillus sp. strain 173 is inhibited in air, due to the production of H_2O_2 (E. Blickstad and G. Molin, submitted for publication). *Lactobacillus* sp. strain 173 was inhibited at 8°C in anaerobic culture (5% CO_2) even though it was isolated from a meat product stored at 4°C in a vacuum package containing a high concentration of CO_2 . This implies that neither the amount of CO_2 nor the temperature used should have been inhibitory. Thus, some unknown factor caused or contributed to the inhibition.

B. thermosphacta and *L. viridescens* consume O_2 at a high rate, leading to an O_2 -restricted environment when growing at high $\mu_{\max}$ values (Blickstad and Molin, submitted for publication). In the present study, the growth rates

were evaluated during the beginning of the log phase and before the culture might have become O_2 restricted. Thus, the $\mu_{\max}$ values reported are only affected by pH and temperature and not by O_2 availability.

B. thermosphacta and *L. viridescens* may be competitive in certain environments. Their growth rates were about the same at 8°C in both aerobic and anaerobic gas atmospheres. In an aerobic environment, high pH (6.3) favored the growth of *B. thermosphacta*, whereas *L. viridescens* grew better at a lower pH (5.8).

B. thermosphacta is unable to grow on beef under anaerobic conditions at pH values below 5.8 (6). It was concluded that this inability depends on inhibition by undissociated lactic acid (10). In the present study, *B. thermosphacta* was unable to grow anaerobically at pH 5.3 in broth. The concentration of undissociated lactic acid originating from the inoculum was below 0.05 mM. Thus, at such a low pH as 5.3 the growth of *B. thermosphacta* may be inhibited by the pH per se.

In the present study, it was demonstrated that the aerobic growth of *B. thermosphacta* was also affected by low pH. The growth rate decreased drastically when the pH was decreased from 5.8 to 5.3. Furthermore, not all of the glucose was consumed at pH 5.3. At the point of inhibition, 56 mM of L-lactic acid had been produced. Since *B. thermosphacta* consumes oxygen, the inhibition may have coincided with a depletion of oxygen; that is, the environment turned anaerobic. At a higher pH (5.6), *B. thermosphacta* is able to grow in air in the presence of 100 mM L-lactic acid (10).

A variety of factors such as pH, temperature, gas environment, and substrate availability affect bacterial growth on meat and meat products. The growth and metabolism of *B. thermosphacta* are highly dependent on these factors as shown by the present and by other studies (8; Blickstad and Molin, submitted for publication). It may change from a neutral spoilage organism producing mainly lactic acid to a potent spoiler producing acetoin, acetic acid, 2,3-butanediol, and fatty acids instead of lactic acid. The most critical factor seems to be the gas atmosphere. In anaerobic culture but not in aerobic culture, L-lactic acid dominated the end products even at 8°C. In air, less lactic acid and more acetoin, acetic acid, and 2,3-butanediol were produced as the pH and temperature decreased.

The importance of the gas atmosphere is confirmed by the study done by E. Blickstad and G. Molin (submitted for publication). With an unlimited O_2 supply, *B. thermosphacta* produces mainly acetoin, acetic acid, and isovaleric acid, whereas under anaerobic conditions, L-lactic acid totally dominates the end products. The

variable amounts of L-lactic acid found in the present study are probably caused by variable degrees of O₂ restriction. A very low lactic acid yield was obtained at 8°C, indicating that this culture was not oxygen limited. By comparing product yields from this culture with the ones obtained from a culture not limited by oxygen at 25°C (E. Blickstad and G. Molin, submitted for publication) it can be concluded that the temperature affected the end product formation. When temperature decreases, more 2,3-butanediol and less acetoin is produced.

High pH increases the yield of heterofermentative end products (other than lactate) of lactic acid bacteria (15). In the present study, *L. viridescens* formed the same type of end products irrespective of both pH and temperature but with various yields, and the heterofermentative end products constituted a fairly constant part of the total product yield (Tables 1 and 2).

It can be concluded that *L. viridescens*, *Lactobacillus* sp. strain 173, and *B. thermosphacta* responded in very different ways to the applied environments. *L. viridescens* had the strongest growth capability, whereas *Lactobacillus* sp. strain 173 was the most susceptible. Both *L. viridescens* and *B. thermosphacta* can be considered as inducible, potent spoilage organisms. *B. thermosphacta* may cause off odors and off flavors due to the production of acetoin, 2,3-butanediol, and isovaleric acid. *L. viridescens* may produce H₂O₂, which causes greening of products. The production of the above-mentioned compounds is stimulated by air (O₂). The significance of *Lactobacillus* sp. strain 173 in spoilage is less certain; perhaps this strain favors long shelf life of meat or meat products.

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Growth and end-product formation of Brochothrix thermos-
phacta ATCC 11509^T and psychrotrophic Lactobacillus spp.
in different gas atmospheres.

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SUMMARY

The effect of different gas atmospheres on the maximum specific growth rate ($\mu_{\max}$) and end-product formation of Brochothrix thermosphacta ATCC 11509^T, Lactobacillus viridescens, Lactobacillus 173 (homofermentative) and Lactobacillus plantarum ATCC 8014^T were studied. The highest $\mu_{\max}$ -values of L. viridescens (0.47 h^{-1}) and B. thermosphacta (0.49 h^{-1}) were found in air. Under anaerobic atmospheres $\mu_{\max}$ was reduced, pure CO_2 giving the greatest reduction. Lactobacillus 173 did not grow in air or pure N_2 . Aerobic growth was accomplished by adding peroxidase while anaerobic growth occurred in the presence of 5-20% CO_2 . Pure CO_2 reduced the growth rate. All test organisms produced mainly lactic acid anaerobically. L. viridescens also produced ethanol while B. thermosphacta produced small amounts of ethanol and formic acid. With oxygen present the number of end-products increased for all organisms. Lactobacillus 173 and L. plantarum produced small amounts of acetic acid and acetoin together with lactic acid. Oxygen induced acetic acid production in L. viridescens and B. thermosphacta. Aerobically B. thermosphacta also produced a large amount of acetoin and smaller amounts of 2,3-butanediol, *i*-valeric acid and *i*-butyric acid. The production of lactic acid by B. thermosphacta was totally blocked during strictly aerobic conditions. All test organisms consumed O_2 during aerobic growth. Hydrogen peroxide was produced by L. viridescens and Lactobacillus 173.

INTRODUCTION

The gas atmosphere surrounding refrigerated proteinous food products is critical for the shelf-life. In air Pseudomonas spp. will dominate and rapidly spoil products such as meat and fish, while Lactobacillus spp. will dominate in CO_2 . This results in a prolonged shelf-life as Lactobacillus are

less deleterious to the quality (Enfors et al. 1979; Blickstad et al. 1981; Molin et al. 1983). Lactobacillus will generally also be the dominating organism in vacuum-packages of muscular foods as well as on cured meat products (Alm et al. 1961; Erichsen & Molin 1981; Blickstad & Molin 1983).

Brochothrix thermosphacta is another "lactic acid bacteria" that has been found on beef, lamb, pork, poultry, cured meat products and fish (Gardner 1981). Both Lactobacillus and B. thermosphacta are associated with spoilage. B. thermosphacta may for example produce acetoin and thus cause off-odours (Stanley et al. 1981) while Lactobacillus may cause souring or greening (Jay 1978).

The present work studies the influence of different gas atmospheres on the growth rate and end-product formation of the homofermentative Lactobacillus SMRICC 173, the heterofermentative Lactobacillus viridescens SMRICC 174 and Brochothrix thermosphacta ATCC 11509^T. All three strains originated from meat or meat products. As a control Lactobacillus plantarum ATCC 8014^T of non-meat origin was included in the study.

MATERIALS AND METHODS

Organisms

The test organisms used were Lactobacillus SMRICC 173 (homofermentative), Lactobacillus viridescens SMRICC 174, Lactobacillus plantarum ATCC 8014^T and Brochothrix thermosphacta ATCC 11509^T. Lactobacillus 173 was isolated from vacuum packaged smoked pork loin stored at 4°C (Blickstad & Molin 1983). L. viridescens was isolated from pork loin stored in 100% CO₂ at 14°C (Blickstad et al. 1981). The characteristics of Lactobacillus 173 and L. viridescens are summarized in Table 1.

Table 1. Characteristics of the Lactobacillus SMRICC 173 and Lactobacillus viridescens SMRICC 174.

Test	<u>Lactobacillus</u> 173	<u>Lactobacillus</u> <u>viridescens</u>
Acid from ¹⁾		
amygdalin	-	-
arabinose	-	-
cellobiose	-	+
fructose	+	+
galactose	+	-
glucose	+	+
lactose	-	-
maltose	-	+
mannitol	-	-
mannose	+	+
melezitos	-	-
raffinose	-	-
rhamnose	-	-
ribose	+	+
sucrose	+	+
trehalose	+	-
xylose	-	-
Hydrolysis of arginine ²⁾	+	-
Gas from glucose ³⁾	-	+
Growth at 15°C (MRS, Oxoid)	+	+

+, positive; -, negative reaction

1) MRS fermentation medium, Harrigan & McCance 1976

2) Collins & Lyne 1970

3) Gibson medium, Gibson & Abdel-Malek 1945

Medium

The growth medium (YTGT) had the following composition (g/l): Yeast Extract (Difco), 24.0; glucose, 20.0; Tryptone (Difco), 5.0; natrium acetate, 1.0; KH_2PO_4 , 0.70; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.52; $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 0.22; sili-

cone anti-foaming agent (30% w/w; BDH Chemicals Ltd, England), 0.1; $\text{MnSO}_4 \times \text{H}_2\text{O}$, 0.06; thiamine-HCl, 0.001. The glucose was autoclaved separately and added aseptically.

Culture equipment

The cultures were performed batchwise in a 1.0 l fermentor (Chemoferm AB, Hägersten, Sweden) supplied with equipment to maintain constant pH, temperature and agitation (400 rpm). The gases used were sterilized using a filter paper (Munktell, Grycksbo Pappersbruk AB, Sweden) and then fed into the medium. The medium was sparged (flow rate 17 l/h) with the applied gas atmosphere for 20 minutes or more before inoculation and then throughout the cultivation. The titration with NaOH (1M), in order to maintain a constant pH, was monitored with a Dose Monitor[®] (Servo Chem AB, Vällingby, Sweden). The dissolved oxygen tension was continuously measured using a galvanic lead-silver electrode. Strictly aerobic conditions (> 87% of saturation) were obtained in a 10 l fermentor (Chemoferm AB) at a stirring speed of 1500 rpm and a gas-flow rate of 180 l/h.

Growth experiments

The strains were stored freeze dried at 4°C. Before inoculation the organisms were subcultured: (i) the freeze-dried culture was transferred into 200 ml YTGT broth and incubated for 7.5 h at 25°C; (ii) 5 ml culture was transferred into 200 ml YTGT broth and incubated for 16.5 h at 25°C before inoculating the fermentor with 20-40 ml. This corresponded to an absorbance (620 nm) of about 0.07 and a cell density of about 4×10^6 cells/ml in the fermentor.

Batch cultures were performed in different gas atmospheres at pH 6.3 and at 25°C. L. viridescens, Lactobacillus 173 and B. thermosphacta ATCC 11509 were cultured in

air, 100% N₂, 100% CO₂, 0.5% O₂ + 20% CO₂ + 79.5% N₂ and 5% CO₂ + 95% N₂. Additionally L. viridescens was cultured in 20% O₂ + 80% N₂. L. plantarum ATCC 8014 was cultured only in air. The gas mixtures, the pure CO₂ and the pure N₂ were purchased from Alfax, Malmö, Sweden and were of the highest purity (>99.999%).

Estimation of growth

Growth was followed by viable count (pour plate technique, APT agar, BBL; incubation at 25°C for 3 days), dry weight (Blickstad & Molin 1981) and absorbance at 620 nm.

The calculation of the maximum specific growth rate ($\mu_{\max}$) was done according to Pirt (1975) and by using the absorbance at 620 nm as a measure of cellmass.

The overall growth yield ($Y_x = \text{g dry weight produced per mol glucose consumed}$) was calculated.

Estimation of product formation

Samples withdrawn from the fermentor were centrifuged (2700 g for 20 min) and the supernatant liquid was stored at -40°C until determination of product formation was carried out.

The concentrations of acetic acid, ethanol, formic acid, glucose, D-lactic acid and L-lactic acid were determined enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, F.R.G.).

The concentrations of acetoin, *i*-butyric acid, *i*-valeric acid and 2,3-butanediol were, after ether extraction (Dainty & Hibbard 1980), determined on a gas chromatograph (Varian 1400). The gas chromatograph was fitted with a 3ft glass column (Chromosorb W-AW, 80-100 mesh and 10% DEGA;

OVSC, Marietta, Ohio) and a flame ionisation detector. The flow rate in the column was 40 ml N_2 /min and in the detector, 30 ml H_2 /min + 190 ml air/min. The temperature in the column was raised from 90 to 140°C (6°C/min). The injector temperature was 150°C and the detector temperature 200°C (the method was described in a personal communication by C. Hibbard, Meat Research Institute, Langford, UK).

The presence of hydrogen peroxidase was detected using Perid[®] strips (detection limit 5 mg/l, Boehringer Mannheim, GmbH-Biochemica, Mannheim, F.R.G.).

The overall product yield (Y_p = mol product formed per mol glucose consumed) was calculated.

RESULTS

Brochothrix thermosphacta ATCC 11509^T

The highest μ_{max} of B. thermosphacta ATCC 11509^T was found in air (Table 2) and the lowest in 100% CO_2 (reduction: 78%). In the other three gas atmospheres the μ_{max} was about the same but lower than in air.

The growth yield drastically increased with the availability of O_2 (Table 2).

B. thermosphacta produced mainly L-lactic acid in anaerobic or O_2 -restricted cultures (Table 3). The oxygen tension in the O_2 -restricted culture went down to about 5% of saturation after about 7 h of cultivation and at a produced dry weight of 0.4 g/l. The oxygen tension remained at this value throughout the culture (24 h).

In strictly aerobic culture no lactic acid or ethanol was produced. Instead significant amounts of acetoin, acetic acid and i-valeric acid were produced.

Table 2. Growth of *Lactobacillus viridescens*, *Lactobacillus* 173, *Brochothrix thermosphacta* ATCC 11509 and *Lactobacillus plantarum* ATCC 8014 in different gas atmospheres on a complex medium with 2% glucose (pH 6.3, 25°C).

Organism	Gas Atmosphere	Maximum. specific growth rate (h ⁻¹)	Final dry weight (g/l)	Growth yield (g biomass/mol glucose consumed)
<u>B. thermosphacta</u>	air*	0.47	6.8	59
	air (O ₂ -restriction)	0.49**	3.0	27
	N ₂	0.20	2.1	18
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	0.19	2.6	18
	5% CO ₂ + 95% N ₂	0.17	2.6	22
	CO ₂	0.11	2.1	18
<u>L. viridescens</u>	air*	0.47	1.4	13
	air (O ₂ -restriction)	0.46**	2.6	23
	N ₂	0.41	2.2	22
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	0.39	2.4	22
	5% CO ₂ + 95% N ₂	0.39	2.5	23
	20% O ₂ + 80% N ₂ (O ₂ -restriction)	0.36**	2.6	23
	CO ₂	0.35	1.7	14
<u>Lactobacillus</u> 173	air	inhibited	0.2	-
	air + peroxidase	0.35	2.5	22
	N ₂	inhibited	0.4	-
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	0.39	2.2	20
	5% CO ₂ + 95% N ₂	0.37	2.6	23
	CO ₂	0.29	2.5	22
<u>L. plantarum</u>	air	0.33	3.5	31

* 10 liter fermentor; air flow 180 l/h; stirring speed 1500 rpm

** evaluated before O₂-restriction

-, not calculated

Table 3. Product formation of *Lactobacillus viridescens*, *Lactobacillus thermosphacta* ATCC 11509 and *Lactobacillus plantarum* ATCC 8014 in different gas atmospheres on a complex medium with 2% glucose (pH 6.3, 25°C).

Organism	Gas atmosphere	Product yield (mol product/mol glucose)									H ₂ O ₂
		D-lactic acid	L-lactic acid	ethanol	acetic acid	2,3-butanediol	1-valeric acid	4-butylric acid	acetoin	formic acid	
<u>B. thermosphacta</u>	air*	0	0	0	0.45	tr.	0.09	tr.	0.73	0.04	-
	air (O ₂ -restriction)	0	1.4	0.08	0.12	0.04	tr.	tr.	tr.	0.08	-
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	0	1.5	0.12	0.02	tr.	tr.	tr.	tr.	0.10	-
	N ₂	0	1.5	0.16	0	0	0	0	0	0.08	-
	CO ₂	0	1.4	0.12	0	0	0	0	0	0.10	-
	5% CO ₂ + 95% N ₂	0	1.5	0.12	0	0	tr.	tr.	0	0.11	-
<u>L. viridescens</u>	air*	0.66	0	0.12	0.54	0	0	0	0	0	+
	air (O ₂ -restriction)	0.88	0	0.58	0.24	0	0	0	0	0	+
	20% O ₂ + 80% N ₂ (O ₂ -restriction)	0.94	0	0.55	0.30	0	0	0	0	0	+
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	1.0	0	0.78	na	0	0	0	0	0	-
	N ₂	1.1	0	0.78	0	0	0	0	0	0	-
	CO ₂	1.0	0	0.78	0	0	0	0	0	0	-
	5% CO ₂ + 95% N ₂	1.1	0	0.86	0	0	0	0	0	0	-
<u>Lactobacillus</u> 173	air + peroxidase	0.28	1.2	0	0.06	0	0	0	0.02	0	+
	0.5% O ₂ + 20% CO ₂ + 79.5% N ₂	0.78	0.80	0	0	0	0	0	0	0	-
	CO ₂	0.74	0.82	0	0	0	0	0	0	0	-
	5% CO ₂ + 95% N ₂	0.88	0.86	0	0	0	0	0	0	0	-
<u>L. plantarum</u>	air	0.88	0.33	0	0.12	0	0	0	0.02	0.29	-

+, more than 5 mg H₂O₂/l; -, less than 5 mg H₂O₂/l; tr., trace amounts below 0.01 mol/mol; na, not analysed

* 10 liter fermentor; air flow 180 l/h; stirring speed 1500 rpm

No effect of the CO_2 -concentration on the end-product formation was noted.

Lactobacillus viridescens

In air L. viridescens had a μ_{max} of 0.47 h^{-1} . This value was reduced by 26% in pure CO_2 (Table 2). Compared to air, μ_{max} was also reduced in an air-atmosphere deficient of CO_2 and in anaerobic atmospheres.

The differences between the growth yields of L. viridescens in the different gas atmospheres were small, except in air or 100% CO_2 where the yield was considerably lower (Table 2).

Besides D-lactic acid, L. viridescens produced ethanol in all atmospheres (Table 3). The presence of O_2 initiated a production of acetic acid which was most pronounced under strictly aerobic conditions. This condition lowered also the amount of ethanol produced. The recorded oxygen tension in the O_2 -restricted cultures reached 0% after about 8 h of cultivation and at a dry weight of 0.8 g/l. Hydrogen peroxide was detected in aerobic cultures.

No effect of CO_2 -concentration was noted on the end-product formation.

Homofermentative Lactobacillus spp.

The highest μ_{max} of Lactobacillus 173 was found in an atmosphere of 5% CO_2 + 95% N_2 or 0.5% O_2 + 20% CO_2 + 79.5% N_2 (Table 2). The lowest μ_{max} was found in pure CO_2 where it was reduced by 24%. In air or in pure N_2 , growth was inhibited after 5 hours at a low dry weight.

The inhibition of Lactobacillus 173 in air was reversed by adding peroxidase (300 U/l) to the medium (Figure 1a). The recorded oxygen tension was reduced in two steps, first down to 50% of saturation during the beginning of the logarithmic growth phase and secondly to 0% at the initiation of the stationary phase. In the presence of peroxidase Lactobacillus 173 grew at the same rate as in 5% CO₂ + 95% N₂ and to the same final dry weight (Table 2). The inhibition in N₂ was reversed by adding 5% CO₂ to the gas phase (Figure 1b).

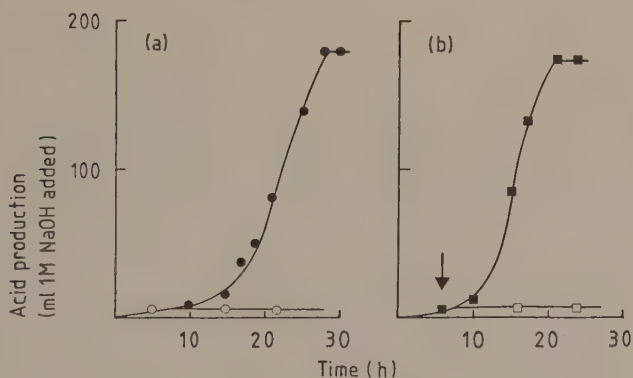


Figure 1. Acid production, expressed in ml NaOH added to maintain a constant pH of 6.3, of Lactobacillus 173 in (a) O, air; ●, air with peroxidase added (b) □, N₂; ■, after the change from N₂ to 5% CO₂ + 95% N₂ at the point indicated by the arrow.

Lactobacillus plantarum ATCC 8014^T had a $\mu_{\max}$ value similar to Lactobacillus 173 but it had a somewhat higher growth yield (Table 2). The recorded oxygen tension was reduced down to 20% at the initiation of the stationary phase.

Lactobacillus 173 anaerobically produced a racemic mixture of lactic acid (Table 3). Aerobically, with per-

oxidase added to the culture, lactic acid was produced together with small amounts of acetic acid and acetoin. The amount of L-lactic acid was under aerobic conditions higher than the amount of D-lactic acid. Detectable amounts of H_2O_2 were found under aerobic conditions in the late logarithmic growth phase.

L. plantarum produced a mixture of L- and D-lactic acid and smaller amounts of acetic acid and acetoin.

DISCUSSION

The present study was performed in a fermentor at 25°C and pH 6.3, i.e. under controlled conditions near growth optimum for the test organisms. These growth conditions were chosen in order to minimize the influence of environmental factors other than the gas atmosphere. Some objections may be raised over a direct application of the results on refrigerated food products. However, general differences in physiological and metabolic features are favourably studied under controlled optimum growth conditions. Often, any differences observed under optimum growth conditions become more distinct under restricted conditions.

All the tested strains except L. plantarum originated from meat or meat products. The homofermentative Lactobacillus 173 belonged to the Lactobacillus Group 1 of Blickstad & Molin (1983). Lactobacillus Group 1 constituted 2-58% of the microflora on cured meat products after storage (Blickstad & Molin 1983). L. viridescens was isolated by Blickstad et al. (1981) from CO_2 -stored pork where it represented 12% of the final microflora. B. thermosphacta ATCC 11509^T which is the type strain of the species, has originally been isolated from fresh pork sausage (McLean & Sulzbacher 1953).

Two features of the present strain of L. viridescens did not agree with the description in Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons 1974), i.e. the organism produced acid from cellobiose and produced D-lactic acid instead of DL-lactic acid. However, considering the fact that the taxonomy of the genus Lactobacillus is often insufficient for strains of meat origin, the resemblance of the present strain to the general description of L. viridescens must be considered as good.

The highest growth rates found for B. thermosphacta, L. viridescens and Lactobacillus 173 were 0.49, 0.47 and 0.39 h^{-1} , respectively (Table 1). These growth rates are lower than some others reported for lactic acid bacteria, e.g. Streptococcus lactis (1.34 h^{-1} , Cogan et al. 1971), Pedococcus pentosaceus (1.27 h^{-1} , Blickstad & Molin 1981), Lactobacillus casei (0.60 h^{-1} , de Vries et al. 1970), but higher than for Leuconostoc cremoris (0.28 h^{-1} , Cooper & Collins 1978).

The inability of Lactobacillus 173 to grow in N_2 was due to the absence of CO_2 (Fig. 1b). CO_2 has been reported as necessary or stimulatory for the growth of several lactic acid bacteria (Lardy et al. 1949; Cogan et al. 1971; Repaske et al. 1974, Blickstad & Molin 1981).

In an atmosphere of low O_2 tension and increased CO_2 concentration (e.g. vacuum-pack; exemplified by the gas containing 0.5% O_2 + 20% CO_2 + 79.5% N_2) both the Lactobacillus 173 and L. viridescens grew at a fairly high rate, while the u_{max} of B. thermosphacta was almost as low as in 100% CO_2 . Thus, in a good vacuum-pack with low oxygen permeability and where the CO_2 increases during storage, the growth of B. thermosphacta seems to be less likely. The film permeability has in fact been demonstrated as being one factor controlling the growth of B. thermosphacta (Campbell et al. 1979).

In the presence of oxygen (20% O_2) L. viridescens and the Lactobacillus 173 produced hydrogen peroxide. The produced H_2O_2 is toxic but may be decomposed by peroxidase (Stanier et al. 1979). In the present study, L. viridescens was able to grow aerobically even when producing H_2O_2 while Lactobacillus 173 was unable to grow in air. This inability to grow aerobically may have been due to the absence of a peroxidase since aerobic growth occurred when peroxidase was added (Fig. 1a).

The production of H_2O_2 is associated with the greening of meat products. L. viridescens is known to cause such greening (Niven & Evans 1957). Hydrogen peroxide can only be produced provided there is oxygen available and this is the case in, for example, vacuum-packages where the film is not totally impermeable to air. However, in a pack where no oxygen exists no H_2O_2 can be produced. On the other hand, if a product that has been stored anaerobically is exposed to air for some days, Lactobacillus spp. established on the product may start to produce H_2O_2 and hence greening may occur.

Oxygen affected the metabolism of B. thermosphacta, L. viridescens and Lactobacillus 173. Thus, acetic acid was only produced aerobically (Table 3). This aerobic production of acetic acid has earlier been shown for other lactic acid bacteria (Keenan 1968; Doelle 1975) and it has been suggested that some lactic acid bacteria may have respiratory activity (Strittmatter 1959; Brown & VanDemark 1968; Doelle 1975). Furthermore it has been suggested that oxygen affects the glucose metabolism on the lactate dehydrogenase level, i.e. pyruvate $\rightarrow$ lactate (Doelle 1975). The present study showed (i) that B. thermosphacta, L. viridescens, Lactobacillus 173 and L. plantarum consumed O_2 at a high rate and down to low O_2 -tension; (ii) that L. viridescens produced acetic acid at the expense of both lactate and ethanol and hence the pathways leading to ethanol production were also affected by the presence of O_2 .

It has been reported that B. thermosphacta produces lactic acid anaerobically but not aerobically (Hitchener *et al.* 1979; Dainty & Hibbard 1980). B. thermosphacta produced, in the present study, no lactic acid under strictly aerobic conditions (Table 3). However, as soon as the O₂-tension decreased to about 5% (saturation of air) the O₂ consumption stopped and lactic acid was produced.

B. thermosphacta aerobically produced a great variety of end-products. Several of these compounds have a repulsive odour. In anaerobic culture $\mu_{\max}$ was relatively low and the number of different end-products were reduced, i.e. the metabolism of B. thermosphacta resembled that of homofermentative lactobacilli. This could mean that provided the O₂-tension is kept low in a food package then B. thermosphacta would not be more destructive to the eating quality than Lactobacillus spp. would. However, other factors than the gas atmosphere could affect the metabolism of B. thermosphacta (Dainty & Hibbard 1980).

It can be concluded that the gas atmosphere affects both the growth rate and metabolism of Lactobacillus viridescens and B. thermosphacta. From other studies it is known that the gas atmospheres in packages of refrigerated proteinous food products select the microflora, often resulting in high numbers of the above organisms (Enfors *et al.* 1979; Blickstad *et al.* 1981; Erichsen & Molin 1981; Molin *et al.* 1983). Thus, it is indicated in the present study that a gas package should not contain any O₂ since it may favour the production of acetic acid, acetoin, short chain fatty acids and H₂O₂. Furthermore the lowest growth rates were found in 100% CO₂. Thus, from a microbiological point of view, an optimal gas-package should contain a high CO₂-concentration but no O₂.

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The effect of water activity on growth and end-product formation of two Lactobacillus spp. and Brochothrix thermosphacta ATCC 11509^T.

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SUMMARY

The effect of water activity (a_w) on the growth and end-product formation of Lactobacillus viridescens SMRICC 174, Lactobacillus SMRICC 173 (homofermentative) and Brochothrix thermosphacta ATCC 11509^T was studied. All strains originated from meat or meat products. The a_w was adjusted in the range 0.94-0.99 with NaCl or glycerol. A greater reduction in growth rates was found for L. viridescens and B. thermosphacta when a_w was regulated with NaCl rather than with glycerol, the opposite was true for Lactobacillus 173. L. viridescens grew at $a_w \geq 0.94$. At 0.94 a_w B. thermosphacta was totally inhibited when NaCl was the solute and Lactobacillus 173 when glycerol was the solute. Only minor variations in the end-product formation of the Lactobacillus spp. were found at different a_w values. In aerobic culture B. thermosphacta produced less L-lactic acid and more acetic acid as the a_w was decreased with NaCl, while the yields were unaffected when glycerol was used.

INTRODUCTION

The water activity, a_w , has a great impact on bacteria. The growth ability is affected and the product formation may also be so (Troller 1980).

Regulation of a_w is used for increasing the shelf-life of special kinds of food products. In intermediate moisture foods a_w is reduced to 0.7-0.9 in order to inhibit any bacterial growth (Troller and Christian 1978). For canned shelf-stable high-moisture foods a combination of pasteurizing heat treatment with a_w adjusted below 0.95 may be used (Leistner et al. 1980). In a study by Blickstad and Molin (1983 a) it was indicated that control of the a_w might be necessary in order to obtain prolonged shelf-life of cured meats, such as frankfurter sausage,

being stored in a controlled gas atmosphere. In cured meat products there is a variation in a_w between different types, depending on; e.g. NaCl concentration and the use of dry ingredients such as milk powder. Since the range of a_w where bacteria normally grow is narrow (0.90 - 1.0; Christian 1980) variations in the formulas affecting a_w may also have a significant effect on the microflora of the products.

Lactobacillus often dominate the microflora of cured meat products (Alm et al. 1961, Mol et al. 1971, Blickstad and Molin 1983 a). Brochothrix thermosphacta has in several cases been found in large numbers on cured meat products (Gardner 1981). Few studies have been made on these significant food spoilers with respect to the effect of a_w . In the present study the growth and end-product formation of two Lactobacillus spp. and B. thermosphacta were determined at different a_w levels. All the strains originated from meat or meat products.

MATERIALS AND METHODS

Lactobacillus viridescens SMRICC 174 (isolated from spoiled pork), Lactobacillus sp. SMRICC 173 (isolated from spoiled smoked pork) and Brochothrix thermosphacta ATCC 11509^T (type strain, isolated from pork sausage) were studied. The Lactobacillus spp. have been characterized by Blickstad and Molin (1983 c).

The cultures were performed batchwise in a 1.0 litre fermentor (Chemoferm AB, Hägersten, Sweden) supplied with equipment for maintaining constant pH (6.3), temperature (25°C) and agitation rate (400 rpm). The gases used were filtered sterile and then fed into the medium. The medium (YTGT, 2% glucose; Blickstad and Molin, 1983 c) was sparged with gas 20 minutes or more before inoculation and then throughout the cultivation. The titration with NaOH

(1M), in order to maintain a constant pH, was monitored with a Dose Monitor[®] (Servo Chem AB, Vällingby, Sweden) and was recorded.

The stock cultures were stored freeze dried at 4°C. Before inoculation the organisms were subcultured twice as described by Blickstad and Molin (1983 c). The inoculate gave an absorbance (620nm) of about 0.07 and a cell density of about 4×10^6 cells/ml in the fermentor.

L. viridescens and Lactobacillus 173 were studied in anaerobic cultures (5% CO₂ + 95% N₂; flow rate 17 l/h) while B. thermosphacta was studied in aerobic cultures (air; flow rate 17 l/h) and anaerobic cultures (5% CO₂ + 95% N₂). The a_w level of the medium was regulated with NaCl or glycerol. The a_w -values applied were 0.98 (3% NaCl or 7% glycerol), 0.96 (6% NaCl or 16% glycerol) and 0.94 (8.5% NaCl or 20% glycerol). The a_w was determined using an electronic hygrometer, Humidat-IC II (Novasina AG, Zürich, Switzerland).

Growth was followed by viable count (pour plate technique, APT agar, BBL, incubation at 25°C for 3 days), dry weight and absorbance at 620nm (Blickstad and Molin 1981) until the titration stopped or as for inhibited cultures until the viable count decreased. The calculation of the maximum specific growth rate (μ_{max}) was done according to Pirt (1975) using the absorbance at 620 nm as a measure of cellmass. The overall growth yield ($Y_x = g$ dry weight produced per mol glucose consumed) was calculated.

A sample withdrawn from the fermentor was centrifuged (5000 rpm, 20 min) and the supernatant liquid was stored at -40°C until determination was carried out. The concentrations of acetic acid, ethanol, glucose, D-lactic acid and L-lactic acid were determined enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, F.R.G.).

The concentrations of acetoin, *i*-butyric acid, *i*-valeric acid and 2,3-butanediol were determined by using a gas chromatographic method (Blickstad and Molin 1983c). The presence of hydrogen peroxide was detected with Perid[®] strips (detection limit 5 mg/l, Boehringer Mannheim, GmbH-Biochemica, Mannheim, F.R.G.). The overall product yield (Y_p = mol product formed/mol glucose consumed) was calculated.

RESULTS

Lactobacillus viridescens SMRICC 174 grew at all a_w values tested, the lowest value being 0.94. The maximum specific growth rates (μ_{max}) at different a_w values are given in Table 1. The μ_{max} varied between 0.05 h^{-1} and 0.39 h^{-1} , the lowest growth rate was found at 0.94 a_w , regulated with NaCl. Adjusting the a_w with NaCl gave a greater reduction in μ_{max} than glycerol (Figure 1a). The growth yield decreased when the a_w was decreased with NaCl (Table 1). When the a_w was reduced with glycerol the growth yield was unaffected by the a_w value applied but was, however, lower than without any glycerol added. The end-product formation was unaffected by the a_w ; D-lactic acid and ethanol being produced (Table 2).

Lactobacillus sp. SMRICC 173 grew at all a_w -values tested except at 0.94 when regulated with glycerol. The μ_{max} values varied between 0.05 h^{-1} and 0.38 h^{-1} (Table 1). The reduction in μ_{max} was greater in the presence of glycerol than NaCl at 0.96 a_w and 0.94 a_w (Figure 1b). The growth yield was practically unaffected except at 8.5% NaCl where a significantly lower yield was found (Table 1). Lactobacillus 173 produced a racemic mixture of lactic acid at all a_w levels regulated with glycerol. The yield of D-lactic acid was lower than the yield of L-lactic acid when NaCl was the solute (Table 1).

Table 1. Growth of Lactobacillus viridescens SMRICC 174, Lactobacillus sp. SMRICC 173 and Brochothrix thermosphacta ATCC 11509^T at different water activities in a complex medium at pH 6.3 and 25°C.

Organism	Gas atmosphere	Solute	Initial a_w	$\mu_{\max}$ (h ⁻¹)	Final dry weight (g/l)	Growth yield (g dry weight/mol glucose consumed)
<u>L. viridescens</u>	5% CO ₂ + N ₂	none	0.99	0.39	2.5	23
		NaCl	0.98	0.26	2.3	20
		NaCl	0.96	0.13	1.5	13
		NaCl	0.94	0.05	0.7	6
		glycerol	0.98	0.33	1.8	15
<u>Lactobacillus</u> 173	5% CO ₂ + N ₂	glycerol	0.96	0.19	1.6	14
		glycerol	0.94	0.12	1.7	16
		none	0.99	0.37	2.6	23
		NaCl	0.98	0.31	2.8	25
		NaCl	0.96	0.17	2.4	22
<u>B. thermosphacta</u>	5% CO ₂ + N ₂	NaCl	0.94	0.05	1.2	11
		glycerol	0.98	0.38	2.4	21
		glycerol	0.96	0.08	2.2	19
		glycerol	0.94	inhibited		
		none	0.99	0.17	2.6	22
<u>B. thermosphacta</u>	air	NaCl	0.98	0.15	2.3	19
		NaCl	0.96	0.07	2.1	18
		NaCl	0.94	inhibited		
		none	0.99	0.49	3.0	27
		NaCl	0.98	0.42	3.4	29
<u>B. thermosphacta</u>	air	NaCl	0.96	0.21	2.9	25
		NaCl	0.94	inhibited		
		glycerol	0.98	0.39	3.4	29
		glycerol	0.96	0.24	2.4	22
		glycerol	0.94	0.08	2.1	18

Table 2. Product yield of *Lactobacillus viridescens* SMRICC 174, *Lactobacillus* sp. SMRICC 173 and *Brochothrix thermosphacta* ATCC 11509^T at different water activities in complex medium at pH 6.3 and 25°C.

Organism	Gas atmosphere	Solute	Initial a _w	Product yield (mol product/mol glucose consumed)							
				D-lactic acid	L-lactic acid	ethanol	acetic acid	acetoin	2,3-butanediol	L-valeric acid	L-butyric acid
<u>L. viridescens</u>	5% CO ₂ + N ₂	none	0.99	1.13	0	0.86	0	0	0	0	0
		NaCl	0.98	0.96	na	0.75	0	0	0	0	0
		NaCl	0.96	1.00	na	0.73	0	0	0	0	0
		NaCl	0.94	1.01	na	0.63	0	0	0	0	0
		glycerol	0.98	0.97	na	0.75	0	0	0	0	0
<u>Lactobacillus</u> 173	5% CO ₂ + N ₂	glycerol	0.96	1.00	na	0.71	0	0	0	0	0
		glycerol	0.94	1.03	na	0.75	0	0	0	0	0
		none	0.99	0.88	0.86	0	0	0	0	0	0
		NaCl	0.98	0.58	1.11	na	0	0	0	0	0
		NaCl	0.96	0.70	1.02	na	0	0	0	0	0
<u>B. thermosphacta</u>	5% CO ₂ + N ₂	NaCl	0.94	0.42	1.27	na	0	0	0	0	0
		glycerol	0.98	0.81	0.76	na	0	0	0	0	0
		glycerol	0.96	0.82	0.84	na	0	0	0	0	0
		none	0.99	0	1.52	0.12	0	0	0	tr.	tr.
		NaCl	0.98	na	1.42	0.13	0	0	0.02	0	0
<u>B. thermosphacta</u>	air	NaCl	0.96	na	1.34	0.14	0	0	0.06	tr.	0
		none	0.99	0	1.42	0.08	0.12	tr.	0.04	tr.	tr.
		NaCl	0.98	na	1.15	0.06	0.27	tr.	0.03	0.02	tr.
		NaCl	0.96	na	0.92	0.08	0.48	tr.	0.08	0.02	tr.
		glycerol	0.98	na	1.31	0.08	0.21	tr.	0.03	0.01	tr.
<u>B. thermosphacta</u>		glycerol	0.96	na	1.33	0.04	0.19	tr.	0.06	0	tr.
		glycerol	0.94	na	1.22	0.06	0.21	tr.	0.04	0.02	tr.
		glycerol		na							

na, not analyzed; tr., trace amounts below 0.01 mol/mol; 0, not detected

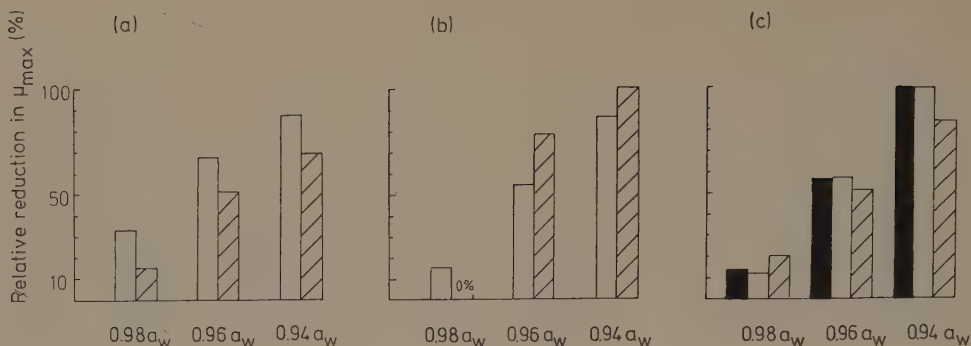


Figure 1. Reduction in μ_{max} of (a) *Lactobacillus viridescens* SMRICC 174 (b) *Lactobacillus* SMRICC 173 and (c) *Brochothrix thermosphacta* ATCC 11509^T at different a_w values relative to 0.99 a_w ; (■) NaCl, aerobic culture; (□) NaCl, anaerobic culture; (▨) glycerol, aerobic culture. *L. viridescens* and *Lactobacillus* 173 were only studied in anaerobic culture.

Brochothrix thermosphacta ATCC 11509^T grew at all a_w values tested except at 0.94 when regulated with NaCl. The μ_{max} varied between 0.07 h^{-1} and 0.49 h^{-1} (Table 1). The reduction in μ_{max} was the same aerobically as well as anaerobically (Figure 1c). Adjusting the a_w with NaCl gave a greater reduction in μ_{max} than glycerol (Figure 1c). The growth yield decreased with decreasing a_w (Table 1). L-lactic acid dominated the end-products at all a_w values applied (Table 2). In addition to L-lactic acid and ethanol *B. thermosphacta* produced small amounts of 2,3-butanediol at $a_w \leq 0.98$ in anaerobic cultures. In aerobic cultures *B. thermosphacta* produced less L-lactic acid while more acetic acid as the a_w was decreased with NaCl. The product yields were unaffected by the a_w when adjusted with glycerol. Low yields of ethanol, 2,3-butanediol, *i*-valeric acid, acetoin and *i*-butyric acid were found in aerobic cultures independently of a_w .

The a_w at the end of the cultivation was slightly

higher than the initial a_w . The increase was on average 0.006 units and independent of whether NaCl or glycerol was used for regulating a_w .

DISCUSSION

The response to low a_w has for several bacterial strains been demonstrated to be dependent on the type of solute used. Some strains are more tolerant to NaCl than to glycerol while others are more tolerant to low a_w when reduced with glycerol (Marshall *et al.* 1971; Troller and Christian 1978; Troller and Stinson 1981). It has been suggested that strains naturally occurring in environments containing NaCl are more tolerant to low a_w adjusted with NaCl (Marshall *et al.* 1971). This hypothesis corresponds with the result of the present study. L. viridescens isolated from native meat was more tolerant when glycerol was used while Lactobacillus 173 isolated from cured meat (2.5% NaCl) was more tolerant to NaCl.

At 0.98 a_w (adjusted with NaCl) the following reductions in growth rates were found as compared to uninhibited cultures: B. thermosphacta, 13%; Lactobacillus 173, 16%; L. viridescens, 33% (Figure 1). The corresponding figures for other lactic acid bacteria are: Lactobacillus plantarum, 18%; Lactobacillus brevis, 27%; Pediococcus pentosaceus, 33%; Leuconostoc mesenteroides, 54% (calculated from Troller and Stinson 1981). Thus the tolerance to a slightly reduced a_w is highly variable among the lactic acid bacteria.

The minimum a_w permitting growth of Lactobacillus and B. thermosphacta is about 0.94 (Brownlie 1966; Christian 1980; present study). Some other minimum a_w values reported for food-borne organisms are: Pseudomonas fluorescens, 0.97; Escherichia coli, 0.95; Pediococcus pentosaceus, 0.95; Salmonella newport, 0.95; Leuconostoc mesen-

teroides, 0.94; Micrococcus sp., 0.83 (Marshall et al. 1971; Troller and Stinson 1981). Thus, B. thermosphacta and Lactobacilli may be considered as organisms being fairly tolerant to low a_w as compared to other food-borne bacteria.

B. thermosphacta did not grow in the presence of 8.5% NaCl while both tested Lactobacillus spp. did. Tolerance to 10% NaCl has earlier been reported for 23 out of 25 food-borne strains of B. thermosphacta, the type-strain not being included (Wilkinson and Jones 1977).

In the presence of 3% NaCl (0.98 a_w) the growth rate of B. thermosphacta was reduced by 13%. Thus, the salt concentrations normally used in cured meat (1.5 - 3%) have alone little inhibitory effect on B. thermosphacta. In fact the spoilage flora of cured meats has in some cases been found to be dominated by B. thermosphacta (Gardner 1981; Blickstad and Molin 1983b).

Very little is known about whether or not or in what way the metabolism of bacteria is affected by a_w . Troller and Stinson (1981) reported that lactic streptococci produced more diacetyl as the a_w was reduced. It has been suggested that adverse conditions increase the heterofermentative character of Lactobacillus spp. (Doelle 1975). In the present study the end-product formation of the heterofermentative L. viridescens was unaffected by the a_w , while the production of L-lactic acid by the homofermentative Lactobacillus 173 was stimulated by NaCl but not by glycerol. Thus, although growing at low a_w values, the lactobacilli did not start to produce any new type of end-product.

In the present experiments it is most likely that the growth of B. thermosphacta in air was O_2 -restricted as judged from the mixture of aerobic and anaerobic end-products formed (Blickstad and Molin 1983c). The growth rates

were evaluated during the beginning of the log phase and before the culture might have become O_2 -restricted. Thus, the $\mu_{\max}$ values reported are only affected by a_w . The end-product formation, on the other hand, was probably affected by both a_w and O_2 -availability.

A clear difference was found in end-product formation for B. thermosphacta in air when comparing NaCl and glycerol adjusted cultures. In the presence of NaCl less lactic acid and more acetic acid was produced as the a_w was lowered (Table 2). Such a change may be explained by an increase in O_2 availability (Blickstad and Molin 1983c). However the yield of acetic acid is unexpectedly high, indicating that the production of acetic acid is enhanced at low a_w values adjusted with NaCl. In the presence of glycerol the yields of lactic acid and acetic acid did not vary with the different a_w values applied and the predominantly anaerobic metabolism was also maintained at low a_w values.

It can be concluded that the effect of water activity was strain and solute dependent. Only minor variations in the end-product formation of Lactobacillus were found at different a_w values, this indicates that these strains spoil meat or meat products in the same way independently of a_w , albeit more slowly at low a_w . The spoilage character of B. thermosphacta was, however, enhanced at low a_w values in the presence of NaCl. This negative effect of low a_w is perhaps avoidable if another method of lowering a_w is chosen.

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